10 Transposable Elements for Insect Transformation

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10.1. Introduction

The ability to create genetically transformed organisms has played a central role in the history of modern genetics, in particular, to our understanding of gene expression and development. Indeed, the pioneering transformation experiments of *Pneumococcus* by Griffith (1928) and subsequent systematic analyses by Avery *et al.* (1944), that showed transformation from a "rough" to a "smooth" bacterial cell wall phenotype, were instrumental in defining DNA as the inherited genetic material. The importance of these initial transformation experiments to prokaryotic genetic analysis was widely appreciated, and continued studies by many other laboratories laid the foundation for modern molecular biology.

The importance of "transformation" technology to eukaryotic genetic studies was apparent, and several of the initial attempts to create transgenic animals were performed in insects, though the means of achieving and assessing insect transformation were not straightforward. The primary reasons why these early attempts to create transgenic insects were largely unsuccessful were the inability to isolate and reproduce individual genetic elements that could be used as transformation vectors and markers, and the lack of efficient means of introducing DNA into germ cells. Most of the initial studies of insect transformation relied on soaking embryos or larvae, with visible mutant phenotypes, in solutions of total wild-type genomic DNA in hopes of reverting the mutant phenotype. The first experiments performed on *Bombyx* and *Ephestia* met with some success where mutant wing color pattern phenotypes were reverted in some organisms, though inheritance was inconsistent and transformation events could not be confirmed unequivocally

(Caspari and Nawa, 1965; Nawa and Yamada, 1968; Nawa *et al.*, 1971). Similar results were obtained in *Drosophila* studies (Fox and Yoon, 1966, 1970; Fox *et al.*, 1970), and for all of these initial experiments, it is most likely that the observed phenotypic changes resulted from extrachromosomal maintainance of introduced DNA in the somatic tissue by an unknown mechanism. A different approach involving the microinjection of wild-type genomic DNA into embryos homozygous for a recessive eye color mutation (*vermilion*), resulted in transformants with a reversion to the normal red eye color phenotype. While the reversion event was genetically mapped away from the mutant locus, a thorough molecular analysis to verify a transformation event, before the lines were lost, was not achieved and so the nature of the phenomenon observed in this experiment remains unexplained (Germeraad, 1976).

In the mid-1970s a turning point in insect science occurred with the extension of molecular genetic analysis to *Drosophila melanogaster*. These early studies and subsequent studies not only provided many of the tools and reagents necessary for developing and critically assessing genetic transformation in insects, but they also emphasized the need for a technology that would facilitate a more complete understanding of the genes being isolated using recombinant DNA methods. One technology that was clearly needed was a means to integrate DNA molecules into the chromosomes of germ cells where it remained stable, resulting in heritable germline transformation. The simple introduction of raw linearized DNA into preblastoderm embryos in the hope of fortuitous recombination into host chromosomes was clearly not reliable. Interest was growing, however, in the use of mobile genetic elements as vectors for DNA integration, including retrotransposons and transposons that were being isolated in *Drosophila* for the first time. Foremost among these was the *P* transposable element, isolated from certain mutant alleles of the *white* gene. The subsequent testing and success of transformation mediated by the *P* element in the *Drosophila* germline proved to be a dramatic turning point in the genetic analysis of an insect species. The eventual impact of this technology on understanding genetic mechanisms in all eukaryotic systems cannot be understated. The success with *P* in *Drosophila* gave hope that this system could be straightforwardly extended to genetic manipulation of other insect species, and especially those highly important to agriculture and human health. While there was reason for optimism, we now realize that this was a naive expectation given what we now understand about the natural history of *P* elements relative to other Class II transposable elements, in particular its extremely limited distribution and its dependence on species-specific host factors. The inability of *P* to function in non-drosophilids, however, was a motivating force to more completely understand transposon regulation and the identification and testing of new vector systems. These included other transposable elements, as well as viral and bacterial vectors.

The development of routine methods for insect gene-transfer was probably delayed by a decade due to attention being focused exclusively on the *P* element. Yet, this delay has resulted in a more varied toolbox of vectors and markers that now allow nearly routine transformation for many important species, and the potential for transformation of most insects (see Handler, 2001). Indeed, some of the tools developed for testing the *P* element, in particular embryonic mobility assays, are now routinely used for initial tests for function of other vectors in an insect species before more laborious and time-consuming transformation experiments are attempted.

The creation of this varied toolbox was first related to the potential need for different vector and marker systems for different insect species. We now realize that the future of genetic analysis will depend on multiple vector and marker systems for each of these species, since genomics and functional genomics studies will require multiple systems for DNA integration and reporters for gene expression. Indeed, germline transformation is essential for the insertional mutagenesis and functional genomics studies that are critical underpinnings for both assessing genomic architecture and relating sequences to gene expression. Notably, the continuing functional analysis of the *Drosophila* genome now relies on the vectors and markers, described in this chapter, that were first developed for nondrosophilid insect species.

10.2. *P* Element Transformation

10.2.1. *P* Element

The use of transposable element-based vectors for *Drosophila* transformation followed the discovery of short inverted terminal repeat-type elements similar to the *Activator* (*Ac*) element discovered in maize by McClinotck (see Federoff, 1989). The first such element to be discovered in insects was the *P* element, the factor responsible for hybrid dysgenesis that occurred in crosses of males from a *P* strain (containing *P* factor) with females from an

M strain (devoid of *P* factor) females (Kidwell *et al.*, 1977). The identification of *P* sequences resulted from the molecular analysis of *P*-induced *white* mutations that occurred in dysgenic hybrids (Rubin *et al.*, 1982). While the initial *P* elements isolated as insertion sequences were incomplete, nonautonomous elements, complete functional elements were later isolated and characterized by O'Hare and Rubin (1983).

P is 2907 bp in length with 31 bp inverted terminal repeats (ITRs) and 11 bp subterminal inverted repeats that occur approximately 125 bp from each terminus (**Figure 1**). Other repeat sequences exist within *P*, but their functional significance, if any, remains unknown. A defining signature for *P*, as with other transposable elements, is the nature of its insertion site which consists of an 8 bp direct repeat duplication. The extensive use of *P* for transformation and transposon mutagenesis has shown the element to have a distinctly nonrandom pattern of integration. It is now clear that *P* elements are blind to a significant fraction of the genome and new gene vectors are being employed in *Drosophila* to complement these limitations. *P* elements and all transposable elements currently used as insect gene vectors belong to a general group of transposable elements known as Class II short inverted terminal repeat transposons (see Finnegan, 1989). These elements transpose via a DNA intermediate and generally utilize a cut-and-paste mechanism that creates a duplication of the insertion site. These are distinguished from Class I elements, or retrotransposons, that have long direct terminal repeats (LTRs) and transpose via reverse transcription an RNA intermediate.

The original use of *P* for germline transformation was accomplished by inserting a marker gene within the element so that it did not disrupt activity of the terminal sequences or the transposase gene. The $rosy^+$ gene was inserted at the 3′ end of the transposase-coding region, but upstream of the 3′ subterminal inverted repeat sequence. Plasmids containing this vector were injected into preblastoderm (syncytial) embryos homozygous for ry^- so the *P* vector could transpose into germ cell nuclei. Germline transformation events were identified in the following generation (G_1) by virtue of reversion of the mutant ry^- eye color phenotype to wild-type. These experiments not only proved the feasibility of transposon-mediated transformation, but also permitted structure–function relationships within the *P* element to be defined (Karess and Rubin, 1984). The *P* transcriptional unit was found to be composed of four exons separated by three introns. Further analysis determined that the *P* transposase function is cell-type specific owing to differential transcript splicing of the third intron that is limited to the germline. The lack of splicing in the soma results in production of nonfunctional truncated polypeptides in this tissue (Rio *et al.*, 1986).

While the original *P* vector allowed efficient transformation, the presence of a functional transposase gene within the vector made the system self-mobilizable (autonomous) and inherently unstable, allowing potential excision or transposition of the original insertion event. Subsequent vector development resulted in a binary system in which the vector transposase was deleted or made defective by insertion of a marker gene. The ability of the transposase

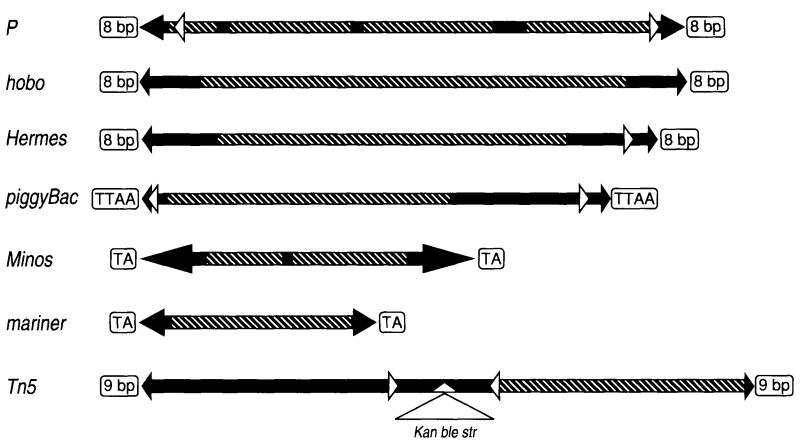

Figure 1 Diagram of transposable elements currently in use for the germline transformation of insect species. The left arms represent the 5′ termini and right arms represent the 3′ termini. Transposon sizes and specific internal elements are shown in relative positions but are not at precise scale. Major structural elements include duplicated insertion sites (open boxes); inverted terminal repeat sequences (black arrowheads); internal subterminal repeat sequences (white arrowheads); transposase coding region (boxed diagonals); and intron sequences (black boxes). The *Tn5* element is a composite transposable element consisting of two functional elements flanking three antibiotic resistance genes. Refer to text for specific details on nucleotide lengths and relative positions.

to act *in trans* allowed transposase to be provided by a separate plasmid (helper), that could facilitate vector integrations when cointroduced with the vector-containing plasmid into the same nucleus (Rubin and Spradling, 1982). Integrations would remain stable if the helper did not integrate, but the original helpers, such as pπ25.1, were autonomous *P* elements themselves that could integrate along with the vector. While helper integration was diminished by injecting much higher concentrations of vector plasmid, this possibility was only eliminated with the creation of defective helpers having one or both of their terminal sequences deleted (known as "wings-clipped" helpers). The first of these was pπ25.7wc, which was immobilized by deletion of 3′ terminal sequences (Karess and Rubin, 1984). This prototype vector system served as a model for binary systems of nonautonomous vector: helper elements used for all the transposon-based transformation systems currently in use (**Table 1**).

A notable characteristic of *P* elements was not only their discontinuous distribution within the species (*P* and *M* strains), but their discontinuous interspecific distribution. Based on its distribution patterns it has become apparent that *P* was recently introduced into *D. melanogaster* from *D. willistoni* by an unknown mechanism (Daniels and Strausbaugh, 1986). Regardless of the mechanism since the 1950s, *P* elements have thoroughly invaded wild populations of *D. melanogaster* (Anxolabéhère *et al.*, 1988), and without the existence of *M* strain laboratory stocks that were removed from nature before this time, the development of *P* vectors might never have been realized. This is due to the repression of *P* mobility in *P*-containing strains that was first observed in hybrid dysgenesis studies, which also showed that movement was not repressed in *M* strains devoid of *P*. The basis for *P* strain repression appears to be due to a number of factors including repressor protein synthesis, transposase titration by resident defective elements, and regulation of transposase gene transcription (Handler *et al.*, 1993b; Simmons *et al.*, 2002). As will be discussed further on, other vector systems in use have thus far been shown to be widely functional in several orders of insects, and the presence of the same or related transposon in a host insect does not necessarily repress vector transposition. In this and several other aspects, the *P* vector system appears to be the exception rather than the rule for transposon-mediated gene transfer in insects.

10.2.2. *P* Vectors and Markers

Regardless of regulatory differences between *P* and other transposon vector systems currently in use, methods developed for *P* transformation of *Drosophila* serve as a paradigm for all other insect vector systems. Those familiar with *Drosophila* transformation will be in the best position to attempt these methods in other insects. Current techniques developed for other insect species are variations on a theme, although as we describe, considerable modifications have been made. Several comprehensive reviews are available for more specific details on the structure, function, and use of *P* for transformation in *Drosophila*, which are highly relevant to the understanding and use of other vector systems (see Karess, 1985; Spradling, 1986; Engels, 1989; Handler and O'Brochta, 1991). Particularly useful are the books and method manuals by Ashburner (1989a, 1989b) that review the various vectors, markers, and methodologies used for *Drosophila* transformation, as well as early techniques used to manipulate *Drosophila* embryos. This information is especially applicable to other insect systems.

The first consideration for transformation is the design of vector and helper plasmids, and the marker system used for transformant selection. The first *P* vectors and helpers were actually autonomous vectors, which was probably a useful starting point since the actual sequence requirements for vector mobility and transposase function were unknown. As noted, the first nonautonomous helper had a 3′ terminal deletion that prevented its transposition, providing greater control over vector stability. However, this source of transposase was inefficient, until it was placed under *hsp70* regulation which allowed transposase induction by heat shock (Steller and Pirrotta, 1986). All other vector system helper constructs have similarly taken advantage of heat shock promoters, mostly from the *D. melanogaster hsp70* gene, but other *hsp* promoters have been tested including those from the host species being transformed. Other constitutive promoters such as those from the genes for *actin* and *α1-tubulin* have proven successful for helper transposase regulation, and will be discussed further on.

While sufficient transposase production is critical for transposition, the structure of the vector is equally important, and for some, very subtle changes from the autonomous vector can dramatically decrease or eliminate mobility. These variations include critical sequences (typically in the termini and subtermini), and placement and amount of exogenous DNA inserted within the termini. For some vectors the amount of plasmid DNA external to the vector can affect transposition rates. Subsequent to the initial test of several *P* vectors, the terminal sequence requirements for *P* mobility were determined to

Table 1 Transposon-mediated germline transformation

Transposon	*Host species*	*Marker*	*Reference*
Hermes	*Aedes aegypti*	*Dm-cinnabar*$^+$	Jasinskiene *et al.* (1998)
		actin5C-EGFP	Pinkerton *et al.* (2000)
	Culex quinquefasciatus	actin5C-EGFP	Allen *et al.* (2001)
	Ceratitis capitata	*Cc-white*$^+$	Michel *et al.* (2001)
	Drosophila melanogaster	*Dm-white*$^+$	O'Brochta *et al.* (1996)
		actin5C-EGFP	Pinkerton *et al.* (2000)
		3xP3-EGFP	Horn *et al.* (2000)
	Stomoxys calcitrans	actin5C-EGFP	O'Brochta *et al.* (2000)
	Tribolium castaneum	3xP3-EGFP	Berghammer *et al.* (1999)
hobo	*Drosophila melanogaster*	*Dm*-mini-*white*$^+$	Blackman *et al.* (1989)
	Drosophila virilis	*Dm*-mini-*white*$^+$	Lozovskaya *et al.* (1996); Gomez and Handler (1997)
mariner (*Mos1*)	*Aedes aegypti*	*Dm-cinnabar*$^+$	Coates *et al.* (1998)
	Drosophila melanogaster	*Dm-white*$^+$	Garza *et al.* (1991); Lidholm *et al.* (1993)
		3xP3-EGFP	Horn *et al.* (2000)
	Drosophila virilis	*Dm-white*$^+$	Lohe and Hartl (1996a)
Minos	*Anopheles stephensi*	actin5C-EGFP	Catteruccia *et al.* (2000b)
	Ceratitis capitata	*Cc-white*$^+$	Loukeris *et al.* (1995b)
	Drosophila melanogaster	*Dm-white*$^+$	Loukeris *et al.* (1995a)
P	*Drosophila melanogaster*	*Dm-rosy*$^+$	Rubin and Spradling (1982)
		Dm-white$^+$	Hazelrigg *et al.* (1984); Pirrotta *et al.* (1985)
		Dm-hsp70-mini-*white*$^+$	Klemenz *et al.* (1987)
		pUChsneo	Steller and Pirrotta (1985)
	Drosophila simulans	*Dm-rosy*$^+$	Scavarda and Hartl (1984)
piggyBac	*Aedes aegypti*	*Dm-cinnabar*$^+$	Lobo *et al.* (2002)
		3xP3-EGFP	Kokoza *et al.* (2001)
	Anastrepha suspensa	PUb-nls-EGFP	Handler and Harrell (2000)
	Anopheles albimanus	PUb-nls-EGFP	Perera *et al.* (2002)
	Anopheles gambiae	hr5-ie1:EGFP	Grossman *et al.* (2001)
	Anopheles stephensi	actin5C-DsRed	Nolan *et al.* (2002)
	Athalia rosae	*Bm*A3-EGFP, *hsp70*-GFP	Sumitani *et al.* (2003)
	Bactrocera dorsalis	*Cc-white*$^+$	Handler and McCombs (2000)
		PUb-nls-EGFP	Handler and McCombs (unpublished data)
	Bombyx mori	*Bm*A3-EGFP	Tamura *et al.* (2000)
		3xP3-EGFP	Thomas *et al.* (2002); Uhlirova *et al.* (2002)
	Ceratitis capitata	*Cc-white*$^+$	Handler *et al.* (1998)
		PUb-nls-EGFP	Handler and Krasteva (unpublished data)
		PUb-DsRed1	Handler and Krasteva (unpublished data)
	Cochliomyia hominivorax	PUb-nls-EGFP	Allen *et al.* (2004)
	Drosophila melanogaster	*Dm-white*$^+$, PUb-nls-EGFP	Handler and Harrell (1999)
		PUb-DsRed1	Handler and Harrell (2001)
		3xP3-EGFP	Horn *et al.* (2000)
		3xP3-EYFP	Horn and Wimmer (2000)
		3xP3-ECFP	Horn and Wimmer (2000)
		3xP3-DsRed	Horn *et al.* (2002)
	Lucilia cuprina	PUb-nls-EGFP	Heinrich *et al.* (2002)
	Musca domestica	3xP3-EGFP	Hediger *et al.* (2000)
	Pectinophora gossypiella	*Bm*A3-EGFP	Peloquin *et al.* (2000)
	Tribolium castaneum	3xP3-EGFP	Berghammer *et al.* (1999) Lorenzen *et al.* (2003)
Tn5	*Aedes aegypti*	3xP3-DsRed	O'Brochta (unpublished data)

EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein.

include 138 bp of the 5′ end and 216 bp of the 3′ end. While the inverted repeat sequences within these terminal regions are identical, the adjacent sequences were found not to be interchangeable in terms of vector mobility (Mullins *et al.*, 1989). Of interest was the discovery that the strongest binding affinity for the *P* transposase was at sequences approximately 50 bp internal to the terminal repeats (Rio and Rubin, 1988; Kaufman *et al.*, 1989). While the minimal sequences required for mobility may be used in vectors, typically the rate of mobility decreases with the decreased length of terminal sequence. Specific sequences may be required for binding of transposase or other nuclear factors, and conformational changes needed for recombination may be dependent upon sequence length and position.

P vector mobility was also found to be influenced by the amount of exogenous DNA inserted between the termini, with transformation frequency diminishing with increasing size. Initial tests with 8 kb vectors marked with *rosy* yielded transformation frequencies of approximately 50% per fertile G_0, while use of 15 kb vectors resulted in 20% frequencies (see Spradling, 1986). Larger vectors could transpose but frequencies approached 1% or less.

Of equal importance to creating an efficient vector system is having marker genes and appropriate host strains that will allow efficient and unambiguous identification or selection of transgenic individuals. Indeed, the genetic resources available for *Drosophila* also provided cloned wild-type DNA and appropriate mutant hosts for use in visible mutant-rescue marker systems that made testing *P* transformation possible. As noted, the first of these used the ry^+ eye color gene, but this required a relatively large genomic fragment of nearly 8 kb. The *white* (*w*) eye color gene was then tested, but this required a genomic sequence that was longer than *ry*, and resultant transformation frequencies were relatively low (Hazelrigg *et al.*, 1984; Pirrotta *et al.*, 1985). New *w* markers, known as mini-*white*, that had the large first intron deleted decreased the marker insert to 4 kb resulting in much more efficient transformation, and placing the mini-*white* marker under *hsp70* regulation increased efficiencies further (Klemenz *et al.*, 1987). Use of *w* markers, especially in CaSpeR vectors (Pirrotta, 1988), has been a mainstay of *Drosophila* transformation, yet expression of the *w* gene in particular is subject to position effect variegation/suppression (PEV) that typically diminishes eye pigmentation. PEV, indeed, was originally discovered as a result of translocating w^+ proximal to heterochromatin (Green, 1996), and it routinely manifests itself in w^- flies transformed with w^+. This effect has been observed with the use of eye pigmentation markers in several other insect species as well.

Other markers based upon chemical selections or enzymatic activity were also developed for *Drosophila*, though none has found routine use. These included alcohol dehydrogenase (*Adh*) (Goldberg *et al.*, 1983) and dopa decarboxylase (*Ddc*) (Scholnick *et al.*, 1983) that complemented existing mutations, and neomycin phosphotransferase (NPT or *neo*) (Steller and Pirrotta, 1985), β-galactosidase (Lis *et al.*, 1983), organophosphorus dehydrogenase (*opd*) (Benedict *et al.*, 1995), and dieldrin resistance (*Rdl*) (ffrench-Constant *et al.*, 1991) which are dominant selections not requiring preexisting mutations (see ffrench-Constant and Benedict, 2000).

10.2.3. *P* Transformation of Non-Drosophilids

Given the straightforward procedures for transforming *Drosophila* with *P* elements, there were high expectations that the system would function in other insects. The ability to test this was facilitated by the development of the neomycin (G418)-resistance marker system (Steller and Pirrotta, 1985), and neomycin resistance-containing *P* vectors were widely tested in tephritid flies and mosquitoes (see Walker, 1990; Handler and O'Brochta, 1991). Unfortunately, the neomycin resistance system was generally unreliable, and recovery of resistant individuals that were not transgenic was common. In three mosquito species, however, neomycin resistant transgenic insects were recovered but they arose from rare transposition-independent recombination events (Miller *et al.*, 1987; McGrane *et al.*, 1988; Morris *et al.*, 1989). Other dominant chemical resistance markers, including *opd* and *Rdl*, which had had some success in *Drosophila*, were also tested, but no transformation events could be verified in other insects. A major limitation of these experiments was that, given the numerous variables involved, it was impossible to determine which components in the system were failing. This limitation led to efforts to determine systematically whether the transposon vector system was, indeed, functional in host embryos, which resulted in the development of rapid transposon mobility assays as described below. The first of these assays tested *P* excision in drosophilid and non-drosophilid embryos, revealing that *P* function decreased in drosophilids as a function of relatedness to *D. melanogaster*, with no function evident in non-drosophilids (O'Brochta and Handler, 1988; Handler *et al.*, 1993a). These results were the first indication that for transposon-mediated germline transformation to succeed in non-drosophilids, new

vector systems would have to be created from existing and newly discovered transposon systems.

10.3. Excision and Transposition Assays for Vector Mobility

Assessing the ability of an insect gene vector to function in a particular species can be challenging. The procedures required to create a transgenic insect using transposable element-based gene vectors require a great deal of technical skill and the ability to perform basic genetic manipulations. Depending on the insect, its generation time, and its amenability to being reared in the laboratory, the process of genetic transformation can be quite lengthy. At the early stages of developing non-drosophilid transformation technology there was little experience in manipulating and injecting the embryos of the various non-drosophilid species of insects. In addition, the genetic markers available to select for, or recognize, transgenic insects were limited and none could be confidently expected to function optimally in the species being tested at that time. Consequently, early efforts to test the functionality of potential gene vectors, by attempting to create transgenic insects, required simultaneous success in dealing with a number of daunting challenges. The failure of these efforts to yield a transgenic insect could not, unfortunately, be ascribed to the failure of any one particular step in the process (see Handler and O'Brochta, 1991). These efforts, therefore, did not represent an isolated test of the gene vector since failure to obtain a transgenic insect might have been due to a failure in DNA delivery, expression of the genetic marker, or the failure of the transposable element vector system. Technology development under these conditions was very difficult. What was needed was an experimental system that permitted the activity of the transposable element system to be assessed in the species of interest independent of any prospective genetic marker system and DNA delivery system. Such a system was developed for investigating the mobility properties of the *D. melanogaster P* element, and was very adaptable to other transposable element and insect systems (**Figure 2**).

The system developed for *P* elements involved transfecting *Drosophila* cells with a mixture of two plasmids – one containing a *P* element inserted into the coding region of the LacZα peptide of a common cloning vector, and a second containing the *P* element transposase gene under the regulatory control

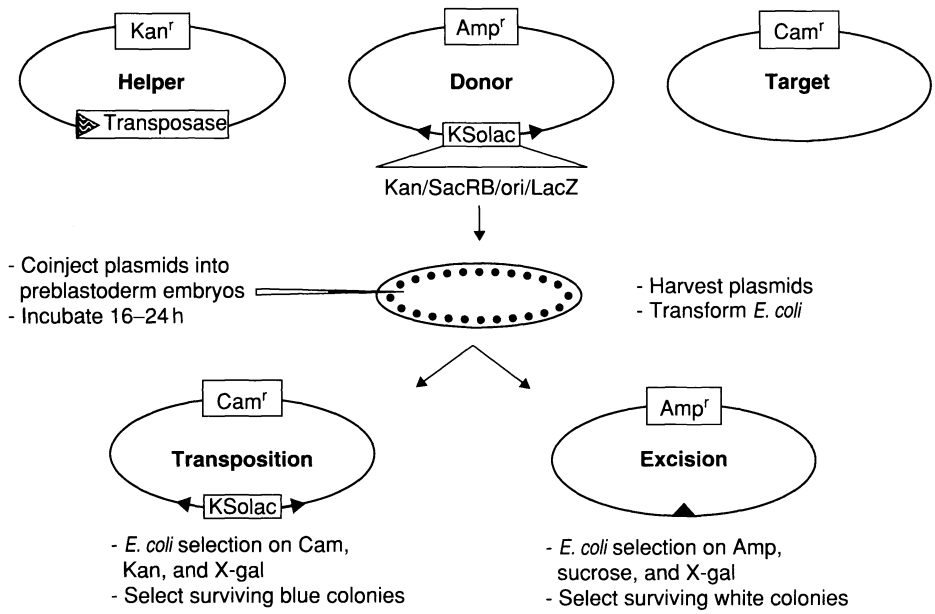

Figure 2 Plasmid-based transposable element mobility assays. A mixture of three plasmids is coinjected into preblastoderm embryos to insure incorporation into nuclei. After approximately 24 h the plasmids are extracted from the embryos and introduced into *E. coli*. Transient expression of the transposase gene on the helper plasmid in the developing embryos results in the production of functional transposase. If the transposase catalyzes excision and transposition of the element, excision will result in the loss of element-specific markers on the donor plasmid. In the example shown sucrose sensitivity, β-galactosidase activity, and kanamycin resistance are lost, and others could be used. Transposition results in the target plasmid acquiring all of the element-specific markers. In this example the target plasmid is from a Gram-positive bacteria and incapable of replicating in *E. coli* unless it acquires the origin of replication present on the element. Assays can be completed in 3 days and rates of movement of 0.001% or greater are routinely detectable.

of a strong promoter (Rio *et al.*, 1986). Transient expression of the transposase gene resulted in the production of transposase, catalyzing the excision of *P* elements from the "excision indicator plasmids." Subsequent recovery of the injected plasmids from the cells, followed by their introduction into an appropriate strain of *Escherichia coli,* permitted plasmids that had lost the *P* element through excision to be recognized by virtue of their restored LacZα peptide coding capacity. This transient *P* element excision assay was readily adaptable to use in *Drosophila* embryos through the process of direct microinjection of preblastoderm embryos, and it played a critical role in assessing the functionality of the *P* element system in a variety of drosophilid and non-drosophilid insect systems (O'Brochta and Handler, 1988).

As originally configured the excision assay only permitted the identification and recovery of excision events that resulted in the restoration of the open reading frame of the LacZα peptide reporter gene. Various modifications in this basic assay were adopted that permitted precise and imprecise excisions to be identified and recovered (O'Brochta *et al.*, 1991). For example, marker genes such as the *E. coli* LacZα peptide coding region, *E. coli supF,* sucrase (*SacRB*) from *Bacillus subtilis*, and streptomycin sensitivity, were incorporated into the transposable element (O'Brochta *et al.*, 1991; Coates *et al.*, 1997; Sundararajan *et al.*, 1999). Plasmids recovered that lacked marker gene expression were usually excision events. Further refinements of the excision assay involved the use of transposable element-specific restriction endonuclease sites as a means for selecting for excision events. Digesting plasmids recovered from embryos with restriction endonucleases with sites only in the transposable element was a very powerful method of physically removing plasmids that had not undergone excision from the pool of plasmids recovered from embryos and used to transform *E. coli*. Each restriction site was essentially a single dominant genetic marker and therefore transposable elements with multiple restriction sites provided a very powerful system for selecting against plasmids that have not undergone excision (O'Brochta, unpublished data).

Continued development of element mobility assays lead to assays in which interplasmid transposition could be measured. These assays involved the coinjection of a transposase–encoding helper plasmid, an element "donor" plasmid and a "target" plasmid. Typically the target plasmid contains a gene whose inactivation results in a selectable phenotype. For example, the *SacRB* gene has been used since its inactivation eliminates sucrose sensitivity. If the donor element also contains unique genetic markers then transposition events would lead to a recombinant plasmid with a new combination of a variety of markers (Saville *et al.*, 1999). Perhaps the most powerful transposition assay developed for assessing transposable elements in insect embryos involved the use of a genetic marker cassette containing a plasmid origin of replication, an antibiotic resistance marker, and the LacZα peptide coding region, in combination with a target consisting of a Gram-positive plasmid (pGDV1) (Sarkar *et al.*, 1997a). pGDV1 contains an origin of replication that cannot function in *E. coli,* although it does have a chloramphenicol resistance gene that is functional in this species. Transposition of the marked transposable element into pGDV1 converts it into a functional replicon in *E. coli*. Because of the absolute *cis*-dependence of origins of replication, and the complete inability of pGDV1 to replicate in *E. coli,* transposition events can be readily detected even at low frequencies.

Transient mobility assays are now a standard for defining vector competence in insect embryos, and in particular, when assessing a vector in a species for the first time. For this application, transposition assays provide the most information relevant to the potential for successful germline transformation, and can be used as a system to test helper construct function. As noted below, however, there may be differing constraints on plasmid and chromosomal transpositions for particular transposons. The use of these assays for analyzing transposon function is discussed in more detail in the relevant sections below.

Embryonic assays also provide an essential test system for assessing potential transgene instability by mobilizing or cross-mobilizing systems within a host genome, which is a critical information for the risk analysis of transgenic insects being considered for release. The importance of excision assays for this purpose became evident by the *hobo* excision assays in *Musca domestica* that revealed the existence of the *Hermes* element (Atkinson *et al.*, 1993), and the subsequent assays that defined the interaction between the two transposons (Sundararajan *et al.*, 1999). Since cross-mobilizing systems do not always promote precise exicisions, assays that reveal imprecise as well as precise excisions are most sensitive for this purpose. Since successful transposition may depend on precise excision, transposition assays may only reveal the existence of mobilizing systems that have a high level of functional relatedness.

10.4. Transformation Marker Systems

The availability and development of selectable marker systems has played a large part in recent

advancements in insect transformation, that have been equal to the importance of vector development. The rapid implementation and expansion of *P* transformation in *Drosophila* was possible, in large part, due to the availability of several eye color mutant-rescue systems. These systems depend on the transgenic expression of the dominant-acting wild-type gene for an eye color mutation present in the host strain (see Sarkar and Collins, 2000). Successful transformation of non-drosophilid species was similarly dependent upon the development of analogous systems, with the first transformations in the medfly, *Ceratitis capitata*, and a mosquito, *Aedes aegypti*, relying on *white* and *cinnabar* mutant-rescue systems. While chemical resistance markers were used initially for non-drosophilid transformation, and can be highly useful for specific applications, their inefficiency and inconsistency when used alone provided ambiguous results for several species (see ffrench-Constant and Benedict, 2000). Eye color marker systems are generally efficient and reliable, and cloned wild-type genes from *Drosophila* often complement orthologous mutant alleles in other insects. However, only a handful of species have stable mutant strains that can serve as suitable hosts for mutant-rescue strategies. The most significant advancement in marker gene development for the wide use of insect transformation has been the development of fluorescent protein markers (Higgs and Sinkins, 2000; Horn *et al.*, 2002). As dominant-acting neomorphs, that do not depend on preexisting mutations, they are directly useful in almost all host strains. When compared to the *white* eye color marker in *Drosophila*, the enhanced green fluorescent protein (EGFP) gene seemed to be less affected by position effect suppression, and thus has the additional advantage of more reliable detection (Handler and Harrell, 1999). Certainly for the forseeable future, fluorescent protein markers will continue to be the markers of choice for most insect transformation strategies.

10.4.1. Eye Color Markers

The first insect transformations used mutant-rescue systems to identify transformant individuals, but in these experiments total genomic DNA was used, rather unreliably, to complement mutations in the respective host strains. The most reliable of these, however, was reversion of the *vermilion* eye color mutation in *D. melanogaster* (Germeraad, 1976). The success of the initial *P* element transformations in *Drosophila* also depended on reversion of eye color mutant strains, but the use of cloned *rosy* and *white* genomic DNA within the vector plasmid allowed for much greater efficiency and reliability. The first non-drosophilid transformations in medfly (Loukeris *et al.*, 1995b; Handler *et al.*, 1998; Michel *et al.*, 2001) similarly relied on use of the wild-type medfly *white* gene cDNA that was placed under *Drosophila hsp70* regulation (Zwiebel *et al.*, 1995). This gene complemented a mutant allele in a *white eye* medfly host strain that was isolated more than 20 years earlier. The medfly *white* gene also complemented the orthologous gene mutation in the oriental fruit fly, yielding in one line a nearly complete reversion (Handler and McCombs, 2000). The first transformations in *A. aegypti* used a kynurenine hydroxylase-*white* mutant host strain, but for these tests the complementing marker was the *D. melanogaster cinnabar* gene (Cornel *et al.*, 1997). The *D. melanogaster vermilion* gene, that encodes tryptophan oxygensase (*to*) has also been used to complement the orthologous *green* eye color mutation in *M. domestica* (White *et al.*, 1996), and the *Anopheles gambiae* tryptophan oxygensase gene complements *vermilion* in *Drosophila* (Besansky *et al.*, 1997). The *vermilion* and *cinnabar* orthologs have also been cloned from *Tribolium*, and while the *white* mutation in this species is complemented by tryptophan oxygensase, no preexisting eye color mutation is complemented by kynurenine hydroxylase (Lorenzen *et al.*, 2002). The use of eye color mutant-rescue systems has certainly been critical to initial advances in insect transformation, and these markers should have continued utility for those species that have been successfully tested. The use of these markers, however, for the development of insect transformation in other species will be limited by the availability of suitable mutant host strains.

10.4.2. Chemical Selections

Previous to the development of mutant-rescue marker systems, transformant selections in non-drosophilid insects focused on genes that could confer resistance to particular chemicals or drugs. Importantly, these types of selections could be used for screening transformants en masse by providing the selectable chemical or drug in culture media. Ideally, only transformant individuals would survive the selection, allowing the rapid screening of large numbers of G_1 insects. For vectors that are inefficient and insects that are difficult to rear, the efficient screening of populations can be essential to identifying transformant individuals. The first drug resistance selection tested used the bacterial neomycin phosphotransferase gene (NPT II or *neomycin*^{r}) that conferred resistance by inactivation of the neomycin analog G418 (or Geneticin) (Steller

and Pirrotta, 1985). This seemed straightforward since the selection and *hsneo* marker system (putting NPT II under heat shock regulation) was already developed and tested in *Drosophila* for mass transformant screens, and the bacterial resistance gene was thought to be functional in most eukaryotes. The initial *P* transformations in *Drosophila* using the pUChsneo vector were generally reliable; however, the marker was not easily transferable to other species. G418 resistance was highly variable, most likely due to species differences in diet, physiology, and symbiotic bacteria, and indeed, variations in resistance in transformed *Drosophila* have been attributed to strains of yeast used in culture media (Ashburner, 1989a). Other chemical resistance markers, including *opd* conferring resistance to paraoxan (Phillips *et al.*, 1990; Benedict *et al.*, 1995), and the gene for dieldrin resistance (*Rdl*) (ffrench-Constant *et al.*, 1991), that were initially tested in *Drosophila* were also problematic when tested in other species. These failures were due in large part to ineffective vector systems, but a common attribute in these studies was the selection of individuals having nonvector related or natural resistance to the respective chemical. While naturally resistant insects could be selected out by molecular tests in primary transformant screeens, the recurrence of resistant insects in subsequent generations would make use of the transgenic strains highly impractical.

While the problems cited made chemical resistance selections frustrating for several species, and they have not been used for any recent transformation experiments, some successes were reported and the need for mass screening still exists. The initial tests for *P* transformation in several mosquito species used the pUChsneo vector with G418 resistant transformants being selected, though transformation frequencies were low and all of them resulted from fortuitous recombination events and not *P*-mediated transposition (Miller *et al.*, 1987; McGrane *et al.*, 1988; Morris *et al.*, 1989). Nonetheless, chemical selections can be very powerful, and if reliable, they would dramatically improve the efficiency of transformation screens for most insects. It is quite possible that many species will not be amenable to current transformation techniques without markers that allow selection en masse. A potential means of increasing the reliability of chemical resistance screens would be to link a resistance marker to a visible marker within the vector. Initial G_1 transformants could be screened en masse by chemical resistance, with surviving individuals verified as transformants and maintained in culture by use of the visible marker. This type of marking is tested by linking the *hsneo* construct with a red fluorescent protein marker in the *piggyBac* vector. Thus far initial results in *Drosophila* are highly encouraging (Handler and Harrell, unpublished data).

Of the enzyme systems tested for chemical selection in *Drosophila* that might be extended to other insects, the *Adh* system might have the most promise (Goldberg *et al.*, 1983). An *Adh* marker gene can complement the *adh* mutation in *Drosophila*, eliminating lethal sensitivity to ethanol treatment in mutant hosts. An *adh* gene has been cloned from the medfly, and a strategy has been developed to use it for genetic sexing by male-specific overexpression (Christophides *et al.*, 2001). Conceivably a similar strategy could be extended to transformant selections, though its use would be limited to medfly and possibly other tephritid species.

10.4.3. Fluorescent Protein Markers

The dramatic advancement of insect transformation in recent years has been due, primarily, to the development of fluorescent protein markers which are dominant-acting neomorphs that do not depend on preexisting mutations. The first of these to be tested was the green fluorescent protein (GFP) gene that was isolated from the jellyfish *Aequorea victoria* (Prasher *et al.*, 1992) and which initially exhibited heterologous function in the nematode *Caenorhabditis elegans* (Chalfie *et al.*, 1994). GFP expression was then tested in transformant *Drosophila* where it was used as a reporter for gene expression (Plautz *et al.*, 1996; Hazelrigg *et al.*, 1998), and several other species for both *in vivo* and *in vitro* studies. GFP was first tested in non-drosophilid insects as marker for Sindbis viral infection in *Aedes aegypti* (Higgs *et al.*, 1996), and the dramatic somatic expression of GFP in adults was highly encouraging for the further use of GFP for germline transformants.

This possibility was first tested in *Drosophila* using a construct that linked EGFP to a *polyubiquitin* promoter and nuclear localizing sequence (Lee *et al.*, 1988; Davis *et al.*, 1995; Handler and Harrell, 1999). A control for transformation, that also allowed a direct comparison of EGFP expression to that from the visible mini-*white* marker, was the initial use of a *piggyBac* vector, pB[Dm*w*, PUbnlsEGFP], that linked the two markers in a mutant *white* strain. The results from this experiment indicated that not only was the PUbnslEGFP marker efficient and easily detectable under epifluorescense optics, but that many of the G_1 transformants that expressed GFP did not express a detectable level

of *white*$^+$. Presumably the chromosomal position effects that suppressed *white* had a negligible effect on GFP expression. This result was highly encouraging for the use of GFP as a marker in non-drosophilids and several subsequent transformation experiments used EGFP regulated by a variety of promoters in *piggyBac, Hermes*, and *Minos* vectors. Notably, this allowed germline transformation to be tested in several species that otherwise have no visible marker systems, such as the Caribbean fruit fly, *Anastrepha suspensa*, which was transformed with pB[PUbnlsEGFP] (Handler and Harrell, 2000). This vector was subsequently tested in the Australian sheep blowfly, *Lucilia cuprina* (Heinrich *et al.*, 2002) and the mosquito *Anopheles albimanus* (Perera *et al.*, 2002). Similarly, a *Hermes* vector marked with EGFP regulated by the *Drosophila actin5C* promoter was first tested in *Drosophila* (Pinkerton *et al.*, 2000), and was used then to efficiently select transformants in *Aedes aegypti* (Pinkerton *et al.*, 2000), *Stomoxys calcitrans* (O'Brochta *et al.*, 2000), and *Culex quinquefasciatus* (Allen *et al.*, 2001). A *Minos* vector marked with *actin5C*-EGFP was used to select *Anopheles stephensi* transformants (Catteruccia *et al.*, 2000b), and a *piggyBac* vector marked with EGFP under *Bombyx actin 3A* promoter regulation was used to transform the lepidopteran species *Bombyx mori* (Tamura *et al.*, 2000) and *Pectinophora gossypiella* (Peloquin *et al.*, 2000).

Both the *polyubiquitin* and *actin* promoters have activity in all tissues throughout development, making insects marked in this fashion particularly useful for some applications such as the marking of insects used in biocontrol release programs (see Handler, 2002b). However, the detection of these markers can be limited due to quenching or obstruction by melanized cuticle or scales, and fluorescent protein expression regulated by a strong tissue-specific promoter can be valuable for difficult insects and particular applications. Foremost among these markers have been a series of fluorescent protein constructs regulated by the artificial 3xP3 promoter derived from the *Drosophila eyeless* gene (Sheng *et al.*, 1997; Horn *et al.*, 2000). The markers express strongly from the larval nervous system and adult eyes and ocelli. A 3xP3–EGFP marker within *piggyBac* was first used to transform *D. melanogaster* and *Tribolium castaneum* (Berghammer *et al.*, 1999) and has since been used in *M. domestica* (Hediger *et al.*, 2000), *Aedes aegypti* (Kokoza *et al.*, 2001), and the sawfly *Athalia rosae* (Sumitani *et al.*, 2003). The particular strengths and weaknesses for a marker construct such as 3xP3–EGFP is evident from experiments where it allowed transformant selection in *Bombyx* embryos prior to larval hatching (Thomas *et al.*, 2002), while it was undetectable in *Aedes aegypti* adults having normal eye pigmentation (Kokoza *et al.*, 2001). It must, therefore, be recognized that the utility of fluorescent protein markers must be considered in the context of the host insect's structure and physiology during development.

GFP expression is less sensitive to position effect suppression than eye color markers, yet there is much evidence for quantitative and qualitative variabilities in fluorescent protein expression from transgenes. It is likely that tissue-specific variations are primarily due to position effects, while expression of new tissue phenotypes are due to proximal enhancer effects. Polyubiquitin-regulated EGFP expression is most intense in the thoracic flight mucles in *Drosophila* and tephritid fruit fly adults. In Caribbean fruit fly transgenic adult lines, EGFP was only observed in the thorax, but spectrofluorometric assays revealed as much as fivefold differences in fluorescence between lines having the same number of vector integrations (Handler and Harrell, 1999), and differences in expression that are stable within lines are often observed by inspection. In contrast to typical thoracic expression in tephritid flies, adult PUb-EGFP expression in *Lucilia* was limited to female ovaries (Heinrich *et al.*, 2002), and a PUb-DsRed transgenic medfly line exhibits most intense expression in the tarsi while another line expresses in abdominal tracheal apertures at the dorsal–ventral midline (Handler and Krasteva, unpublished data). In *Tribolium*, 3xP3–EGFP expresses typically from the eyes and brain, though several lines are atypical with one having muscle-specific expression throughout development (Lorenzen *et al.*, 2003). In *Anopheles stephensi*, the 3xP3–EGFP marker showed atypical expression in the pylorus, epidermal cells and in a subset of cells in the rectum (O'Brochta, Kim, and Koo, unpublished data).

The use of GFP will certainly be continued for transformant identification in many, if not most, other species where transformation is tested. Yet continuing studies in species already transformed will require multiple marking systems that are distinguishable from one another, especially when coexpressed. This will allow the detection of multiple independent transgenes when used in concert for conditional gene expression systems and gene discovery methods such as enhancer traps (Bellen *et al.*, 1989; Wilson *et al.*, 1989; Brand *et al.*, 1994). After testing 3xP3–EGFP, the 3xP3 promoter was linked to the GFP redshifted variants that emit blue (BFP), cyan (CFP), and yellow (YFP) fluorescence; these were

tested in *Drosophila*, and have proven useful individually as reporters and for identifying transformants (Horn and Wimmer, 2000). BFP and GFP have distinct enough emission spectra to be used together, though BFP photobleaches quickly and is not useful for many applications. While use of EGFP with ECFP is also problematic, ECFP and EYFP can be distinguished when using appropriate filter sets. For details on appropriate filter sets for particular applications see Horn *et al.* (2002) and the website for Chroma Technology Corp. (Chroma, 2004) which manufactures filters for most of the stereozoom fluorescence microscopes used for insect studies.

The most spectrally distinct fluorescent protein distinct from GFP and its variants is a red fluorescent protein, known as DsRed, isolated from the Indo-Pacific sea coral *Discosoma striata* (Matz *et al.*, 1999). It was first tested in insects by linking it to the *polyubiquitin* promoter in a *piggyBac* vector (pB[PUb-DsRed1]) and tested in *Drosophila*, where it exhibited highly intense expression (Handler and Harrell, 2001). Importantly, DsRed expression was completely distinguishable from EGFP when the two transgenic lines were interbred, and when coexpressed as an hsp70-Gal4/UAS-DsRed reporter in lines having vectors marked with EGFP. DsRed has since been incorporated into several mosquito and fruit fly species (Nolan *et al.*, 2002; Handler, unpublished data).

Both EGFP and DsRed are highly stable and generally resistant to photobleaching, and could be detected in tephritid flies several weeks after death, though DsRed1 was relatively the more stable of the two. This is highly advantageous for the use of these genes as markers for released insects that might only be retrieved several weeks after death in traps. A drawback for fluorescent proteins, and DsRed in particular, is that they require oligomerization and slow maturation that can take up to 48 h, resulting in low intensity in early development. However, variants of DsRed with shorter maturation times (Campbell *et al.*, 2002), and new fluorescent proteins with enhanced properties for specific applications are becoming available on a consistent basis (see Matz *et al.*, 2002).

10.4.3.1. Detection methods for fluorescent proteins Once heterologous expression of GFP in nematodes was discovered it was realized that use of the marker for whole-body analysis of gene expression would require an optical system allowing a large depth of field and a stage with working space for culture plates. Up to this time, most epifluoresence systems were linked to compound or inverted microscopes that had limited field depth and capability to manipulate organisms under observation. This led to the development of an epifluorescence module using a mercury lamp that could be attached to a Leica stereozoom microscope system. Most major microscope manufacturers now market integrated epifluorescent stereozoom microscopes with capabilities for several filter systems.

A lower cost alternative for GFP screening is use of a lamp module using ultra bright blue light emitting diodes (LEDs) with barrier filters that attaches to the objective lens of most stereozoom microscopes (BLS Ltd., Budapest, Hungary). It costs considerably less than a mercury lamp system, but at present, it only has capabilities for detecting GFP and YFP.

The use of fluorescent protein markers, and especially multiple markers will be greatly aided by the use of fluorescence activated embryo sorters. A device first developed to sort *Drosophila* embryos expressing GFP (Furlong *et al.*, 2001) has been modified and commercially marketed for *Drosophila* and other organisms by Union Biometrica (Somerville, MA, USA). The latest sorting machines are highly sensitive having the ability not only to distinguish different fluorescent proteins, but also to discriminate between levels of fluoresence from the same protein. Thus, these systems may have enormous importance to the straightforward screening for transgenics, and more sophisticated assays such as those for enhancer traps. Practical applications could include the screening of released transgenic insects caught in traps (in systems adapted for adults), or for genetic-sexing of embryos having a Y-linked or male-specific fluorescent marker.

10.5. Transposon Vectors

10.5.1. *Hermes*

10.5.1.1. Discovery, description, and characteristics *Hermes* is a member of the *hAT* family of transposable elements and is related to the *hobo* element of *D. melanogaster*, the *Ac* element from maize, *Zea mays*, and the *Tam3* element from *Antirrhinum majus* (Warren *et al.*, 1994). The initial interest in this family of elements by those interested in creating new insect gene vectors stemmed from two observations. First, during the middle and late 1980s the mobility characteristics of the *Ac/Ds* element system were being extensively studied because the element was recognized as having great potential to serve as a gene-analysis and gene-finding tool in maize and other plants. In addition, the mobility properties of *Ac/Ds* were being extensively tested in species of plants other than maize and in almost

every case evidence for *Ac/Ds* mobility was obtained (Fedoroff, 1989). *Ac/Ds* appeared to be a transposable element with a very broad host range, unlike, for example, the *P* element from *D. melanogaster*, which only functions in closely related species (O'Brochta and Handler, 1988). Because transposable elements with broad host ranges were of interest to those attempting to develop insect transformation technology, *Ac*-like elements warranted attention. The second significant observation at this time was that the *hobo* element from *D. melanogaster* had notable DNA sequence similarity to *Ac/Ds*, suggesting that it was a distant relative of this broadly active element (Calvi *et al.*, 1991). Investigation into the host range of *hobo* using plasmid-based mobility assays (as described above) ensued (O'Brochta *et al.*, 1994). It was during the investigation of *hobo* that *Hermes* was discovered (Atkinson *et al.*, 1993). Atkinson *et al.* (1993) performed plasmid-based *hobo* excision assays in embryos of *M. domestica* as part of an initial attempt to assess the host range of *hobo*. Assays were performed in the presence of *hobo*-encoded transposase and *hobo* excision events were recovered suggesting that *hobo*, like *Ac/Ds*, would have a broad host range. However, when the assays were performed without providing *hobo*-encoded transposase *hobo*, excision events were still recovered in *M. domestica*. The movement of *hobo* in the absence of *hobo*-transposase was completely dependent upon the inverted terminal repeats of *hobo* and the resulting excision events had all of the characteristics of a transposase-mediated process. It was proposed that *M. domestica* embryos contained a *hobo* transposase activity and that this activity arose from the transposase gene of an endogenous *hobo*-like transposable element (Atkinson *et al.*, 1993). These investigators were eventually able to confirm their hypothesis and the element they discovered was called *Hermes* (Warren *et al.*, 1994).

Hermes is 2749 bp in length and is organized like other Class II transposable elements in that it contains ITRs and a transposase-coding region (**Figure 1**). It contains 17 bp ITRs with 10 of the distal 12 nucleotides being identical to the 12 bp ITRs of *hobo*. *Hermes* encodes for a transposase with a predicted size of 72 kDa and based on the amino acid sequence is 55% identical and 71% similar to *hobo* transposase (Warren *et al.*, 1994). The cross-mobilization of *hobo* by *Hermes* transposase that was proposed by Atkinson *et al.* (1993) was tested directly by Sundararajan *et al.* (1999). These investigators used plasmid-based excision assays in *D. melanogaster* embryos to show that *hobo* transposase could mobilize *Hermes* elements and that *Hermes* transposase could mobilize *hobo* elements (Sundararajan *et al.*, 1999). The phenomenon of cross-mobilization has important implications for the future use of transposable element-based gene vectors in non-drosophilid insects and will be discussed below. As is typical of transposable elements, *Hermes* is present as a middle repetitive sequence within the genomes of multiple strains of *M. domestica* and in all populations examined there appeared to be full-length copies of the element. The natural history of this element within *M. domestica* has not been investigated and its mobility properties within this species and the existence of any regulatory system remain unknown.

10.5.1.2. Patterns of integration The integration behavior of *Hermes* has been examined in a variety of contexts. Sarkar *et al.* (1997a, 1997b) tested the ability of *Hermes* to transpose, using a plasmid-based assay, in five species of Diptera. They recovered transpositions of *Hermes* in the target plasmid at a frequency of approximately 10^{-3} in all species tested. In addition they examined the distribution of 127 independent transposition events into the 2.8 kb plasmid used as a target in their assay and observed a distinctly nonrandom pattern of integrations. Most notable was the existence of three sites that were targets for *Hermes* integration 10 or more times each. In an experiment in which any site used two or more times was considered a hot spot for integration, the three sites used 10 or more times constitute sites with unusual characteristics. The precise nature of those characteristics however could not be defined. The sites shared four of eight nucleotides of the target site in common (GTNNNNAC); however, other sites with this nucleotide composition were not equally attractive as integration sites indicating that other factors must be influencing target choice. Saville *et al.* (1999) demonstrated that sequences flanking *hobo* integration hot spots were critical for determining the targeting characteristics of a site. These investigators were able to move an 8 bp *hobo* target site from plasmid to plasmid without losing its target characteristics as long as they included 20 bp of flanking sequence on each side of the target. It was suggested that proximity to a preferred integration site increased the likelihood of a site being used as a target (Sarkar *et al.*, 1997a). They found that sites 80 and 160 bp flanking the integration hot spot were also preferred integration sites. The authors suggested that nucleosomal organization of the target contributes significantly to the target site selection process and contributed to the local juxtaposition of hot spots and flanking DNA.

10.5.1.3. Structure–function relationships Many Class II transposable elements contain a distinct amino acid motif within their catalytic domains consisting of two aspartate residues and a glutamate. This DD35E motif can be found in many but not all Class II transposable elements. The presence of this motif in *Hermes* transposase has been unclear. Bigot *et al.* (1996) proposed the existence of a DDE motif among members of the *hAT* family. However, they proposed that the second aspartate was replaced by a serine in *Ac*, *hobo*, and *Hermes*. Capy *et al.* (1996) concluded that *hAT* elements, like *P* elements from *Drosophila*, do not contain the DDE motif based on sequence alignments, and Lerat *et al.* (1999) supported this conclusion based on the lack of similarity in predicted secondary structure of the transposase of members of the *mariner/Tc* superfamily and *hobo* transposase. Michel *et al.* (2002) examined experimentally the importance of D402, S535, and E572 to the proper functioning of *Hermes* transposase. They found that mutations D402N and E572Q abolished transposase activity while the mutations S535A and S535D had no effect on transposase activity. The work of Michel *et al.* (2002) provided the first experimental data to support the hypothesis that the positive charge of residues D402 and E572 are required for transposition. The authors concluded, based on these data, that D402, S535, and E572 do not constitute the catalytic center of *Hermes* transposase because one of the residues was not essential for activity. Therefore, *Hermes* (and *hAT* elements in general) do not appear to be of the DD35E type of transposable elements, making them distinct from *mariner/Tc* elements.

Because *Hermes* transposase acts within the nucleus it is expected to contain a nuclear localization signal to direct the mature transposase from the ribosome to the nucleus. Deletion and site-directed mutagenesis analysis were performed that demonstrated that the *Hermes* nuclear localization signal is located at the amino acid end of the protein and divided among three domains (Michel and Atkinson, 2003).

The ITRs of transposable elements play an essential role in their mobility. Altering the sequence of ITRs can, depending on the element, lead to loss of function, hyperactivity of the element, or switching the mode of transposition from a cut-and-paste mechanism to a replicative mechanism. *Hermes* contains imperfect ITRs with a 2 bp mismatch within the ITR (Warren *et al.*, 1994). In addition, a naturally occurring polymorphism in the terminal nucleotide of the right 3′ ITR exists. Elements with a cytidine in the terminal position of the right ITR have no activity within *D. melanogaster* but are capable of undergoing an aberrant form of transposition in mosquitoes. Small pentanucleotide motifs in the subterminal regions of both *Hermes* and *hobo* have been found to be important for the mobilization of *Hermes* and *hobo*. The sequences GTGGC and GTGAC are interspersed throughout the subterminal region of the element, and similar repeats are present in the subterminal regions of *Ac* and are known to be transposase binding sites. In *Hermes*, altering a single repeat can eliminate transpositional activity (Atkinson *et al.*, 2001).

Hermes transposase is capable of dimerizing and one region of the protein critical for dimerization is located in the C-terminus of the protein including amino acids 551–569. This region is not only essential for dimerization but is also required for transposition activity. A second region that affects dimerization is located in the N-terminus of the protein within the first 252 amino acids of the transposase. However, this region apparently plays a nonspecific role in dimerization (Michel *et al.*, 2003).

10.5.1.4. Host range of *Hermes* *Hermes* has a wide insect host range and has been found to function (as measured by either plasmid-based mobility assays or by germline transformation) in at least 13 species of insects including 11 flies, one beetle, and one moth (Atkinson *et al.*, 2001). *Hermes* functions rather efficiently in *D. melanogaster* and transforms this species at rates of 20–40% (O'Brochta *et al.*, 1996). In all other species tested the efficiency of transformation was considerably lower and tended to be less than 10%. For example, *T. castenaeum* was transformed at a rate of 1%, *A. aegypti* at 5%, *C. quinquefasciatus* at 11%, *C. capitata* at 3%, and *S. calcitrans* at 4% (Atkinson *et al.*, 2001). In all insects except mosquitoes, *Hermes* appeared to use a standard cut-and-paste type mechanism as is typical of most Class II transposable elements. Such integrations are characterized by the movement of only those sequences delimited by the ITRs, and the integrated elements are flanked by direct duplications of 8 bp. Integration of *Hermes* into the germline of *A. aegypti* and *C. quinquefasciatus* appears to occur by a noncanonical mechanism resulting in the integration of DNA sequences originally flanking the element on the donor plasmid. The amount of flanking DNA that accompanies the integration of *Hermes* in these mosquito species varies. In some cases two tandem copies of the *Hermes* element were transferred to the chromosome and each copy was separated by plasmid DNA sequences (Jasinskiene *et al.*, 2000). Although

these transposition reactions are unusual they are dependent upon *Hermes* transposase since the introduction of *Hermes*-containing plasmid DNA in the absence of *Hermes* transposase failed to yield transformation events. The germline integration behavior of *Hermes* in mosquitoes is not unique; however, other elements being used as gene vectors such as *mariner* and *piggyBac* have occasionally shown similar behavior in *A. aegypti* (O'Brochta, unpublished data). Transposition assays performed with plasmids in developing mosquito embryos and in mosquito cell lines showed that *Hermes* could transpose via a canonical cut-and-paste type mechanism under these conditions (Sarkar *et al.*, 1997b). The basis for the difference in types of integration events between plasmid-based transposition assays and chromosomal integrations is unknown but may reflect differences in somatic and germ cells. In *Aedes*, canonical cut-and-paste transposition has been readily detected in the somatic tissues of insects containing an autonomous element. Germline transposition in these same insects has not been detected. It has been suggested that mosquitoes might contain endogenous *hAT* elements that affect the ability of *Hermes* elements to be integrated precisely. An alternative suggestion is that *Hermes* may have a second mode of transposition as do the transposable elements *Tn7*, *IS903*, and *Mu*, which utilize a replicative mechanism of integration. Such a mechanism would result in integration products that resemble those observed in the germline of *A. aegypti* and *C. quinquefasciatus*. Replicative transposition of *Hermes* has not been demonstrated experimentally and direct tests of the "alternate mechanism" hypothesis have not been reported.

10.5.1.5. Postintegration behavior Once integrated into the genome of *D. melanogaster*, *Hermes* maintains its ability to be remobilized and has shown mobility characteristics that are similar to other transposable elements. Following the introduction of an autonomous *Hermes* element in which the transposase gene was under *hsp70* promoter regulation, and also contained an EGFP marker gene under constitutive regulatory control of the *actin5C* promoter, Guimond *et al.* (2003) found that the element continued to transpose in the germline at a rate of 0.03 jumps per element per generation. The element used in this study was also active in the somatic tissue and they used this as a means of collecting approximately 250 independent transposition events. Analysis of somatic integration events revealed a number of interesting patterns. First, they found that transpositions were clustered around the original integration event. On average 39% of the *Hermes* transpositions recovered were intrachromosomal and 17% were within the same numbered polytene chromosome division. Ten percent of the new insertions were at sites within 2 kb of the donor element, indicating that *Hermes*, like other transposable elements, shows the characteristic of local hopping. Local hopping refers to the tendency of some elements to preferentially integrate into closely linked sites. Local hopping has been described for a number of elements and is likely to be a general characteristic of Class II transposable elements although the mechanistic basis for this behavior is unknown. Certain regions of the *D. melanogaster* genome, as defined by numbered divisions of the polytene chromosomes, are preferred as integration sites, with these regions being repeatedly targeted by *Hermes*. The observed clustering of independent transposition events in regions of the chromosome seems to reflect undefined aspects of the transposition process that might be influenced by the chromatin landscape. With one exception, the clustering observed by Guimond *et al.* (2003) was not correlated with any common feature of the chromosomes or the genes within a region. This type of nonrandom pattern of integration with regional differences has also been reported for other elements. Interestingly, there does not seem to be any strong correlation between the preferred insertion-site regions of the elements *P*, *hobo*, and *Hermes*, at least with respect to chromosome 3 of *D. melanogaster* (see figure 7 from Guimond *et al.*, 2003). Guimond *et al.* (2003) also observed a notable clustering of integrations in polytene chromosome division 5. Eight of the 11 integration events recovered from division 5 (3.2% of all the transposition events examined) were within the 2.7 kb segment of DNA upstream of the cytoplasmic actin gene, *actin5C*. This same 2.7 kb segment of the 5′ regulatory region of *actin5C* was also present within the autonomous *Hermes* element, as a promoter for the EGFP marker, which the investigators tracked as it jumped within the genome.

The strong clustering of transpositions in a target sequence that is homologous to a sequence contained within the vector has been referred to as "homing." This type of target site selection bias was first described for *P* elements and has been reported on a number of occasions. It was initially reported as a strong bias in the integration site distribution of a number of primary germline integration events in which a *P* element containing the *engrailed* gene preferentially integrated into the *engrailed* region of the host genome (Hama *et al.*, 1990; Kassis *et al.*, 1992). A similar biasing of integration site selection was also observed with *P* elements containing *Bithorax*

and *Antennapedia* regulatory sequences (Engstrom *et al.*, 1992; Bender and Hudson, 2000). Taillebourg and Dura (1999) reported a remarkable example of homing of a remobilized *P* element in *D. melanogaster*. This element contained either an 11 kb or 1.6 kb fragment of the 5′ region of the *linotte* gene, and it was found that 20% of the remobilized elements integrated into the 5′ region of the *linotte* gene. Insertions in this case were highly localized and most occurred within a 36 bp fragment of the *linotte* regulatory region. *Hermes* homing indicates that the phenomenon is not element specific, but may be a general characteristic of Class II elements. Guimond *et al.* (2003) suggested that homing was a special case of local hopping, and the physical proximity between donor elements and target sites seems to underlie the phenomenon of local hopping. The presence of transgene regulatory sequences (e.g., *actin5C* 5′ region) may promote tethering of the donor elements to similar regulatory regions via proteins with common DNA binding sites. Deliberate tethering or transposable elements to selected sequences may be a means to regulate target site selection and to minimize the detrimental mutagenic effects of transposable element integration (Bushman, 1994; Kaminski *et al.*, 2002).

The postintegration behavior of the same autonomous *Hermes* element described above in *A. aegypti* had quite different characteristics. In this case germline transposition of the autonomous *Hermes* element was never detected, and it should be noted that the primary integration events in the germline involved the integration of DNA sequences flanking the element (Jasinskiene *et al.*, 1998, 2000). Despite the fact that the element was intact and that functional transposase was expressed, the element was immobile in the germline. This was not the case, however, in the soma of *A. aegypti* where *Hermes* excision and cut-and-paste transpositions were readily detected. Transposition events in the soma had all of the hallmarks of Class II cut-and-paste integration. Only those sequences precisely delimited by the ITRs moved and integration resulted in the creation of 8 bp direct duplications at the target site. Excision of *Hermes* was imprecise and led, in some cases, to the creation of small deletions. The basis for the difference in behavior of the *Hermes* element in the germline versus the somatic tissue of *A. aegypti* is unknown. Clearly the postintegration behavior of *Hermes* in this species will influence how this element will be employed, and in situations were germline stability is essential, *Hermes* will be particularly useful. It will not be useful in its present form for constructing gene-finding tools such as enhancer and promoter traps that rely heavily on transposable element vector remobilization to be effective.

10.5.1.6. Extrachromosomal forms of *Hermes* Excision of *Hermes* in *M. domestica*, and autonomous *Hermes* integrations in *D. melanogaster* and *A. aegypti*, lead to the formation of circularized *Hermes* elements in which the terminal inverted repeats are jointed end-to-end in various ways following the excision reaction (Atkinson and O'Brochta, unpublished data). The most common configuration results in the ends being joined end-to-end with a short spacer sequence between them. The spacer sequence was most often 1, 3, or 4 bp but could also be as much as 200 bp. The extrachromosomal *Hermes* elements found in *M. domestica* are particularly interesting because they have been found in all populations tested and in great abundance in somatic tissue. These data provide evidence for the somatic activity of *Hermes* in the insect from which it was originally isolated. Circularized forms of excised transposable elements of a number of types have been reported in the past (Sundraresan and Freeling, 1987). For example, circularized forms of *Ac/Ds* have been described as well as *Minos* (Arca *et al.*, 1997; Gorbunova and Levy, 1997), yet the significance of extrachromosomal forms of transposable elements has remained unclear. In some cases the circularized elements do not contain intact ITRs and consequently the elements are not expected to be integration competent. Based on rather limited data it has generally been concluded that such forms represent byproducts of aborted or interrupted transposition reactions. A study of the extrachromosomal forms of *Hermes* suggests that these elements may have some biological significance. Circularized *Hermes* elements with intact ITRs are integration-competent, potentially allowing them to contribute to forward transposition (Atkinson and O'Brochta, unpublished data). The ability of circularized forms of excised *Hermes* elements to reintegrate may have an impact on the transmission potential of this element. The existence of a large pool of functional extrachromosomal transposable elements may have implications for the ability of the element to be transferred horizontally and may provide an additional means of vertical transmission (e.g., maternally inherited), both of which will potentially enhance the elements ability to increase in frequency within a population. The biology of extrachromosomal *Hermes* elements needs to be investigated further.

10.5.1.7. *hAT* elements have been found in other insects The Queensland fruit fly, *Bactrocera tryoni*, contains members of at least two distinct *hAT*-like transposable elements (Pinkerton *et al.*, 1999). *Homer* is a 3789 kb element whose sequence is 53% identical to *Hermes* and 54% identical to *hobo*. The transposase coding region is approximately 53% identical and 71% similar to the transposases of *Hermes* and *hobo*. Similarly, the ITRs of *Homer*, which are 12 bp in length, are identical to those of the *hobo* and *Hermes* elements at 10 of 12 positions. There are also *Homer*-like elements within *B. tryoni*. There are fewer than ten copies per genome, and while these elements have not been fully characterized, a conceptual translation of the transposase of this *Homer*-like element reveals 48% identity and 66% similarity to the transposase of *Homer*. These *Homer*-like elements are as similar to *hobo* as they are to *Homer*. Although *Homer* appears to be weakly functional in *D. melanogaster* based on plasmid-based excision assays, all *Homer*-like elements contain inactivating frameshift mutaions.

The blowfly *L. cuprina* contains a nonfunctional *hAT* element called *hermit*. *Hermit* was initially found by low stringency hybridization screening of an *L. cuprina* genomic library using a DNA probe homologous to *hobo* (Coates *et al.*, 1996). *Hermit* is 2716 bp and contains perfect 15 bp ITRs, the distal 12 of which are identical to the *hobo* ITRs at 10 of 12 positions. Although inactive because of frameshift mutations within the transposase coding region, its amino acid sequence is 42% identical and 64% similar to *hobo* transposase. *Hermit* is unusual in that it is present as a unique sequence within *L. cuprina*, in contrast to multiple copies that exist for most transposons. Although present only once within this species it does appear to have arisen within the genome as a result of transposition since the existing copy of the element is flanked by an 8 bp direct duplication of a sequence that is similar to the consensus target site duplication derived from other *hAT* elements. *Hermit* appears to have become inactivated soon after integrating into the *L. cuprina* genome.

Several *hAT* elements have been discovered in tephritid fruit flies using a polymerase chain reaction (PCR) approach similar to that used to discover *Hermes* (Handler and Gomez, 1996). Of these elements, a complete *hAT* transposon (*hopper*) was isolated from a genomic library of the wild Kahuku strain of the Oriental fruit fly, *B. dorsalis*, using the *B. dorsalis hobo*-related element (Bd-HRE) PCR product as a hybridization probe (Handler and Gomez, 1997). A complete 3120 kb element was isolated having 19 bp ITRs. However, the putative transposase-coding region was frameshifted and it did not have a duplicated 8 bp insertion site, suggesting that it had accumulated mutations and was nonfunctional. The Kahuku sequence was used to isolate additional *hopper* elements using an inverse and direct PCR approach, and a new 3131 bp *hopper* was isolated from the *B. dorsalis white eye* strain (Handler, 2003). This element has an uninterrupted coding region and an 8 bp duplicated insertion site. Notably, *hopper* is highly diverged from all other known insect *hAT* elements and its transposase is distantly yet equally related to the coding regions of *hobo* and *Ac*. Of the terminal 12 nucleotides only five are identical to those of *hobo*, while six are identical to the ITRs of *Homer* (*B. tryoni*). *hopper* also exists in the melonfly, *B. cucurbitae*, and another *hAT* element originally discovered in the melonfly, that is closely related to *hobo* and *Hermes*, also exists in *B. dorsalis* (Handler and Gomez, 1996).

hAT elements have been also reported in the human malaria vector *A. gambiae*. Approximately 25 copies of sequences that resemble *hAT* transposases were discovered although none appeared to be part of an intact transposable element. More recently, however, search criteria were used based on unique aspects of *hAT* transposable elements such as length and spacing of ITRs and the characteristics of *hAT* element target sites. This search revealed a *hAT* element in *A. gambiae* that contained perfect 12 bp ITRs flanked by 8 bp direct duplications and a 603 amino acid transposase open reading frame that appeared to contain no internal stop codons. This element (*Herves*) is most closely related to *hopper* and the ability of this element to excise and transpose in *A. gambiae* or other species has not been determined (Atkinson and Arenburger, personal communication).

10.5.2. *piggyBac*

10.5.2.1. Discovery of *piggyBac* and other TTAA-specific elements Similar to several other insect transposable element systems, the *piggyBac* element was discovered fortuitously in association with a mutant phenotype. However, unlike all the other transposons used for insect transformation, the mutant phenotype was the result of a functional element that had transposed into an infectious organism. Fraser and colleagues (see Fraser, 2000) discovered several Few Polyhedra (FP) mutations in the baculoviruses, *Autographa californica* nucleopolyhedrovirus (AcNPV), and *Galleria mellonella* nucleopolyhedrovirus (GmNPV), after passage through the *Trichoplusia ni* cell line TN-368 (Fraser *et al.*, 1983, 1985). Among these elements that inserted specifically into tetranucleotide TTAA sites

was *piggyBac* (then named IFP2), which transposed into AcNPV. Although it might be assumed that IFP2 was an autonomous functional element based on its mobility, another TTAA insertion-site element, *tagalong* (then called TFP3), discovered in AcNPV and GmNPV, was later found not to have a transposase coding region and thus had to be mobilized by another TFP3 or related element. Autonomous functional elements have not yet been found for *tagalong*, though the original IFP2 *piggyBac* element was indeed functional (Wang *et al.*, 1989; Wang and Fraser, 1993). All the *piggyBac* elements discovered in TN-368 were found to be identical, having a length of 2472 kb with 13 bp perfect ITRs and 19 bp subterminal repeats located 31 bp from the 5′ ITR and 3 bp from the 3′ ITR (Cary *et al.*, 1989) (**Figure 1**). The transposase coding region exists as a single reading frame of 2.1 kb that encodes a protein with a predicted molecular mass of 64 kDa. The functionality of *piggyBac* and the precise nature of its transposition was further verified by a series of viral and plasmid transposition and excision assays. A *piggyBac* indicator plasmid was marked with *polh/lacZ*, and assays in the fall armyworm, *Spodoptera frugiperda* cell line SF21AE showed that the original *piggyBac* element, within the p3E1.2 plasmid, could mobilize the marked element. These assays proved that the 3E1 *piggyBac* element encoded a functional transposase, and defined the element's TTAA insertion-site specificity and the precise nature of its transposition. Importantly, these assays also showed directly that *piggyBac* could be mobilized in other lepidopteran species (Fraser *et al.*, 1995), indicating that it might function similarly as a vector for germline transformation. This was a critical realization given the failure of *P* to be mobilized in non-drosophilids, which was consistent with its failure as a vector in these species.

10.5.2.2. *piggyBac* transformation The failure of *P* vectors to transform non-drosophilid species made the testing of other available transposon systems a high priority. The other systems found to be functional in non-drosophilids, however, were first tested successfully for gene transfer vector function in *Drosophila*. For *piggyBac*, germline transformation was first attempted in the Mediterranean fruit fly, *Ceratitis capitata*. This was possible due to the availability of a marker system that had been tested previously by medfly transformation with the *Minos* transposon vector. The medfly *white* gene cDNA was linked to the *Drosophila hsp70* promoter, and was used as a mutant-rescue system in a white eye host strain (Loukeris *et al.*, 1995b; Zwiebel *et al.*, 1995). In the absence of data for the minimal sequence requirements for *piggyBac* mobility, the first *piggyBac* vector was constructed by insertion of the 3.6 kb *hsp-white* cDNA marker into the unique *Hpa*I site within *piggyBac* in the p3E1.2 plasmid. None of the *piggyBac* sequence was deleted though the insertion interrupted the coding region eliminating transposase function. Construction of the first helper was a simple deletion of the 5′ ITR sequence resulting from a *Sac*I digestion and religation of p3E1.2. There is some uncertainty as to whether the upstream *Sac*I site cuts within the *piggyBac* promoter (Cary *et al.*, 1989), yet transposase expression was indeed sufficient to support germline transpositions from the vector plasmid. The first experiment with this helper in the medfly resulted in one transgenic line at a transformation frequency of 5% per fertile G_0. However, sibling sublines exhibited two and three independent integrations (Handler *et al.*, 1998). This experiment with a *piggyBac*-regulated helper was repeated with five additional G_1 lines isolated, but at approximately the same frequency. These attempts at *piggyBac* transformation yielded relatively low transformation frequencies, but it was notable that a lepidopteran transposon vector system had autonomous function in a dipteran species.

Subsequent to the medfly transformation, *piggyBac* transformation was tested in *Drosophila* using the mini-*white* marker from that species (Handler and Harrell, 1999). Using the self-regulated helper, transformants were isolated at a similar frequency of 1–3%, but tests with a *hsp70*-regulated transposase increased the frequency to above 25%, consistent with *P* and *hobo* transformations using heat shock promoted transposase.

Given that *piggyBac* was first isolated from a lepidopteran species, there was some optimism that it would be functional as a vector in other moth species. Function was first tested by transposition assays in the pink bollworm, *Pectinophora gossypiella* (Thibault *et al.*, 1999), which then led to successful germline transformation of this species using the phspBac helper and a vector marked with EGFP regulated by the *Bombyx actinA3* promoter (Peloquin *et al.*, 2000). Concurrent experiments were also performed in the silkmoth *B. mori* using a similar *actinA3*-regulated EGFP marker, but for this species transformation was achieved with an *actinA3*-regulated transposase helper (Tamura *et al.*, 2000). While these are the only moth species reported to be transformed with *piggyBac*, several other dipteran species have been transformed, as well as species in the orders Coleoptera and Hymenoptera. The dipteran species transformed include several of medical and agricultural importance, such

as the mosquitoes *Aedes aegypti* (Kokoza *et al.*, 2001; Lobo *et al.*, 2002), *A. gambiae* (Grossman *et al.*, 2001), *A. albimanus* (Perera *et al.*, 2002), and *A. stephensi* (Nolan *et al.*, 2002), and the tephritid fruit flies *Anastrepha suspensa* (Handler and Harrell, 2000) and *Bactrocera dorsalis* (Handler and McCombs, 2000). Other transformed dipterans include *M. domestica* (Hediger *et al.*, 2000) and *L. cuprina* (Heinrich *et al.*, 2002). Of particular importance at this time, has been the use of *piggyBac* to transform a coleopteran, the red flour beetle, *Tribolium castaneum* (Berghammer *et al.*, 1999; Lorenzen *et al.*, 2003), and a hymenopteran, the sawfly *Athalia rosae* (Sumitani *et al.*, 2003). Notably, all of these species were primarily transformed using a helper regulated by the *Drosophila hsp70* promoter, and with vectors marked with EGFP, though other fluorescent proteins have since been used for some as well.

Although most of these transformations occurred at frequencies between 3% and 5% per fertile G_0, dramatic differences between species have been observed as well, and in some of the same species performed by different laboratories. A single transformant line was reported for *Anopheles gambiae*, at a frequency of approximately 1% (Grossman *et al.*, 2001), while transformation in *A. albimanus* occurred at frequencies ranging from 20% to 40% (Perera *et al.*, 2002). The first transformations of *Tribolium* occurred at an unusually high frequency of 60% (Berghammer *et al.*, 1999).

Many of the transformations were preceded by testing *piggyBac* function by embryonic transposition assays that were first developed for *piggyBac* mobility in the pink bollworm (Thibault *et al.*, 1999). As discussed previously, these assays can rapidly assess the relative mobility of *piggyBac* in a specific host species in a few days. Positive results from these assays provided some assurance that more tedious and time-consuming transformation experiments had some likelihood of success. For some studies the assays also were used to test promoter function in helper plasmids, or provided insights into insertion site specificity, or determined the likelihood of a particular vector construct retaining function in the absence of specific sequences (Lobo *et al.*, 2001). For example, *piggyBac* helper promoters were tested by transposition assays and germline transformation in *D. melanogaster* and *L. cuprina* (Li *et al.*, 2001a; Heinrich *et al.*, 2002). It was found that in *Drosophila*, an *hsp70*-regulated helper yielded the highest transposition frequency, while a constitutive *α1-tubulin*-regulated helper was more effective for germline transformation. By comparison, in *Lucilia* the *hsp70* helper was most effective for both plasmid and germline transpositions, while the *Drosophila α1-tub* helper failed to support transformation. Transposition assays have also shown target site preferences among the TTAA sites within the pGDV1 target plasmid, and assays in *Drosophila* indicated a bias for sites having A or T nucleotides at positions −3, −1, +1, and +3 relative to TTAA (Li *et al.*, 2001a). However, a sequence analysis of 45 genomic integrations sites in *Tribolium*, after *piggyBac* vector remobilization, failed to show this bias (Lorenzen *et al.*, 2003), which may be an indication of species specificity for insertion site preference.

Mobility assays also provide a rapid means of testing sequence requirements for vector mobility, which allow modifications for more efficient vector function. Since vector mobility is known to be affected negatively with increasing size, this information should allow minimal vectors to be created that retain optimal function. However, minimal sequence requirements for plasmid transpositions may differ from those for chromosomal transposition. For example, excision and transposition assays performed in *Trichoplusia ni* embryos showed that the *piggyBac* inverted terminal repeat and subterminal repeat sequences were sufficient for transposition (35 bp from the 5′ terminus and 63 bp from the 3′ terminus), but that an outside spacer region between the ITRs of greater than 40 bp is necessary for optimal transposition from a plasmid (Li *et al.*, 2001b). Use of similar vectors in *Drosophila*, however, did not result in germline transformants (Handler, unpublished data). The minimal sequence requirements for *piggyBac* transformation verified thus far for *Drosophila* are 300 bp from the 5′ terminus and 250 bp from the 3′ terminus (Li, Fraser, and Handler, unpublished data).

10.5.2.3. Phylogenetic distribution of *piggyBac* and implications for transgene stability Unlike most other transposons used for transformation, *piggyBac* is not an apparent member of a larger family, or superfamily of related elements such as the *mariner/Tc* or *hAT* families. Until recently, the only *piggyBac* elements known were the functional elements orginally discovered in *T. ni* (Fraser *et al.*, 1983). Thus, an unexpected finding from the Southern analysis of *B. dorsalis* transformants was that 8 to 10 *piggyBac*-related elements exist in the host strain genome (Handler and McCombs, 2000). PCR analysis of internal coding sequence indicated that these were nearly identical elements, though none has been found to be identical to *piggyBac*, nor are their coding regions consistent with transposase functionality. The isolation of complete *B. dorsalis*

piggyBac elements as genomic clones and by inverse PCR indicate that complete elements exist with conserved terminal and subterminal sequences that are integrated into duplicated TTAA insertion sites. *Bactrocera dorsalis* is part of a larger species complex and PCR analysis indicated that, indeed, *piggyBac* exists throughout the complex having nucleotide sequence identities of 92% among all the elements sequenced, with individual identities of 96–99% to one another and the *T. ni* 3E1 *piggyBac* (Handler, unpublished data).

10.5.2.4. *piggyBac* in other insects The evolutionary distance between *T. ni* and *Bactrocera* strongly suggests that the transposon moved between these species by recent horizontal transmission, and the separation of their geographical habitats raises the possibility that this movement may have been mediated by intermediary species. A Southern blot survey for *piggyBac* in more than 50 species showed the most clear evidence for multiple *piggyBac* elements in *S. frugiperda*, but hybridization patterns suggested that most of the elements are defective and nonfunctional (Handler, unpublished data; see Handler, 2002a). Evidence for *piggyBac* in other insects and other organisms, including mosquitoes and humans, comes from recent sequence data from genome projects. Although discrete sequence similarities suggests that *piggyBac* has an ancient history, there is little evidence at present to indicate that *piggyBacs* have coevolved as functional elements, and related complete elements have yet to be discovered.

The first functional *piggyBac* elements were discovered in a *T. ni* cell line, but little analysis has been done to characterize *piggyBac* in the organismal genome. Recent hybridization analysis of *piggyBac* from larval *T. ni* genomes indicates that *piggyBac* exists, and that its general structure is consistent with full-length functional elements. However, a PCR survey of these genomic sequences has only identified nonfunctional elements, having a level of identity no greater than many of the elements found in *Bactrocera* (>96%) (Zimowska and Handler, unpublished data). If functional *piggyBacs* do not exist *in vivo*, this could be explained by genomic instability that may arise from a highly active transposon. Functional elements may create a genetic load resulting in organismal lethality, which is more easily withstood in cell lines. If this is the case, it is therefore intriguing to consider how the functional element arose in the cell line.

It is also intriguing to consider how horizontal transmission of *piggyBac* may have occurred, considering that the element was originally discovered by virtue of its transposition into an infectious baculovirus. This could potentially explain a distribution among lepidopterans, but not the apparent recent movement between moths and flies. Understanding the interspecies movement of *piggyBac*, as well as all other vectors used for practical application, will be critical to undertanding and eliminating risk associated with the release of transgenic insects.

10.5.3. *mariner*

10.5.3.1. Discovery, description, and characteristics The *mariner* element was first discovered as an insertion element responsible for the *white*-peach (w^{pch}) mutant allele of *D. mauritiana* (Haymer and Marsh, 1986; Jacobson *et al.*, 1986). This particular allele was interesting when discovered because it was highly unstable with reversions to wild type occurring at a frequency of approximately 10^{-3} per gene per generation. *white*-peach individuals also had a high frequency of mosaic eyes, at an approximate frequency of 10^{-3}, suggesting somatic instability. Molecular analysis of the w^{pch} allele indicated that it was the result of a 1286 bp transposable element insertion into the 5′ untranslated leader region of the *white* gene (Jacobson *et al.*, 1986) (**Figure 1**). The *mariner* element is a Class II type transposable element with 28 bp imperfect inverted repeats with four mismatches. The element recovered from w^{pch} contained a single open reading frame capable of encoding a 346 amino acid polypeptide (Jacobson *et al.*, 1986). While the original w^{pch} was highly unstable, another strain of *D. mauritiana* was discovered in which mosaicism of the eyes occurred in every fly (Bryan *et al.*, 1987). This mosacism factor was found to be heritable and was referred to as *Mos1* (Mosaic eyes). *Mos1* was a dominant autosomal factor on chromosome 3 and was subsequently found to be identical to *mariner* except for six amino acid differences in the putative transposase coding region (Medhora *et al.*, 1988). *Mos1* encodes for a functional transposase while the 346 amino acid polypeptide of the w^{pch} *mariner* element was not a functional transposase.

One of the most notable characteristics of *mariner* and *mariner*-like elements (*MLE*s) is their widespread distribution. *MLE*s are found not only in insects and invertebrates but also in vertebrates and plants (Robertson, 2000; Robertson and Zumpano, 1997). Not long after the *D. mauritiana mariner* elements were described, a related element was discovered in the *cecropin* gene of the moth *Hyalophora cecropia* (Lidholm *et al.*, 1991). Based on the sequence comparison between the *mariner* elements from *D. mauritiana* and *H. cecropia*, Robertson (1993) designed degenerate PCR primers

and surveyed 404 species of insects for the presence of related sequences. He found that 64 of the genomes examined contained *MLE*s, and within this group are five subgroups referred to as the *mauritiana, cecropia, mellifera, irritans*, and *capitata* subgroups (Robertson and MacLeod, 1993). Since that original analysis insect *MLE*s have continued to be discovered and currently there are two additional subgroups recognized, known as *mori* and *briggsae* (Lampe *et al.*, 2000). Additional subgroups are likely to be recognized in the future as additional representatives of this family of elements are found. Elements from different subgroups are typically about 50% identical at the nucleotide sequence level while the transposases encoded by elements from different subgroups are usually between 25% and 45% identical at the amino acid level. A notable feature of the phylogenetic relationships of the *MLE*s is their incongruence with the phylogenetic relationships of the insects from which they were isolated. The implication is that many of these elements were introduced into their host genome via a horizontal gene transfer event (Robertson and Lampe, 1995a). The abundant examples of horizontal transfer of *mariner* elements have led to the conclusion that such transfers occur relatively frequently. Hartl *et al.* (1997) estimated that the rate of horizontal transmission of *MLE*s is about the same as the rate of speciation, at least within the *D. melanogaster* species subgroup. The widespread occurrence of horizontal transmission of *MLE*s has been proposed to be critical for the long-term survival of these elements. Horizontal transmission provides a means for invading naive genomes where element proliferation can occur before inactivating influences of mutation and host regulation can occur (Hartl *et al.*, 1997).

Although hundreds of *MLE*s have been reported, only two (*Mos1* from *D. mauritiana* and *Himar1* from *Haematobia irritans*) have been demonstrated to be functional or active. *Haemotobia irritans* contains approximately 17 000 copies of *Himar1*, although all of the copies examined were highly defective. Functional elements could be reconstructed based on the consensus sequence of *Himar1* and then constructed by modifying the closely related *Cpmar1* element from the green lacewing, *Chrysoperla plorabunda,* to match the *Himar* consensus sequence (Robertson and Lampe, 1995b; Lampe *et al.*, 1998). Purification of the transposase from a bacterial expression system and its use in an *in vitro* mobility assay demonstrated the functionality of the *Himar1* protein and the ITRs of the element (Lampe *et al.*, 1996).

10.5.3.2. Structure–function relationships The transposases of *MLE*s belong to a large group of integrases and transposases that share a significant feature of their catalytic domains. Specifically, *MLE*s contain the highly conserved DD35E motif within the active site of the protein (Robertson, 2000). This part of the active site interacts with a divalent cation that is essential for catalysis. Transposase binds to the ITRs of the element, and gel retardation assays were used to assess the binding activity of eight mutant transposases with deletions at the N- or C-termini (Auge-Gouillou *et al.*, 2001a). It was possible to show that amino acids 1–141 were sufficient for binding to the ITRs. The ITR binding domain of *Mos1* transposase differs somewhat from that of *Tc1* elements in that it is composed of two different structural motifs, a helix–turn–helix motif and an α-helical region (Auge-Gouillou *et al.*, 2001a).

The ITRs of *Mos1* are not identical and differ in sequence at four positions, which have effects on the activity of the element *in vitro*. Auge-Gouillou *et al.* (2001b) reported a 10-fold higher affinity of *Mos1* transposase for the 3′ ITR compared to the 5′ ITR. In addition, modified 5′ ITRs that were made to resemble 3′ ITRs at one of the four variable positions resulted in an increase in transposase binding. These investigators also showed that a *Mos1* element with two 3′ ITRs had 104 times the transposition activity of the native ITRs (Auge-Gouillou *et al.*, 2001b). This hyperactive double-ended configuration has not been tested *in vivo*.

Hyperactive transposase mutants of the *Himar1* transposase have been reported (Lampe *et al.*, 1999) and one of the mutants contains two amino acid changes (at positions 131 and 137) in the ITR binding domain of the protein. Although not tested directly, it is possible that these hyperactive mutants result in increased binding of the transposase and consequently higher rates of movement. Paradoxically, neither *Himar1* nor any of the hyperactive muatants shows any transpositional activity in insects (Lampe *et al.*, 2000).

10.5.3.3. Host range of *mariner* The widespread distribution of *MLE*s in nature and the frequent examples of their horizontal transfer between species suggest that these elements have a broad host range. Empirical studies in which *Mos1* has been employed as a gene vector in a wide variety of organisms supports this conclusion. *Mos1* has been used successfully to create transgenic *D. melanogaster* (Lidholm *et al.*, 1993), *D. virilis* (Lohe and Hartl, 1996a), and *Aedes aegypti* (Coates *et al.*,

1998). In each of these species the frequency of transformation was approximately 5%. This element has also been used to create transgenic *B. mori* cells in culture (Wang *et al.*, 2000). In addition to transgenic insects, *Mos1* has been used to create transgenic *Leishmania* (Gueiros-Filho and Beverley, 1997), *Plasmodium* (Mamoun *et al.*, 2000), zebrafish (Fadool *et al.*, 1998), and chickens (Sherman *et al.*, 1998). Similarly the *Himar1* element has been shown to function in *E. coli* (Rubin *et al.*, 1999), Archaebacteria (Zhang *et al.*, 2000), and human cells (Zhang *et al.*, 1998). However, this element has not been shown to be active in *D. melanogaster* or any other insect species, for reasons that are not clear (Lampe *et al.*, 2000).

10.5.3.4. Postintegration behavior The postintegration behavior of *Mos1* has been investigated in *D. melanogaster* and *A. aegypti*. *mariner* gene vectors used to create transgenic *D. melanogaster* have been found to be uncommonly stable even in the presence of functional transposase. Lidholm *et al.* (1993) created two lines of transgenic *D. melanogaster* with a *mariner* vector derived from *Mos1* and containing the mini-*white* gene as a genetic marker. When these lines were crossed to *Mos1* transposase-expressing lines, eye mosaicism was found in only 1% of the progeny, while these same *Mos1* expressing lines resulted in 100% mosaicism of the w^{pch} element. Similarly, germline transposition occurred at rates of less than 1% (Lidholm *et al.*, 1993), and Lohe *et al.* (1995) reported similar evidence for postintegration stability of *mariner* vectors. Lozovsky *et al.* (2002) suggested, after investigating the postintegration mobility of a number of *mariner* vectors containing different genetic markers in different locations within the element, that *mariner* mobility is highly dependent upon critical spacing of subterminal sequences and ITRs. They found that vectors with simple insertions of exogenous DNA of varying lengths and in varying positions showed levels of somatic and germline excision that were at least 100-fold lower than that observed with uninterrupted *mariner* elements. Only vectors consisting of two, almost complete, elements flanking the marker gene showed detectable levels of both somatic and germline mobility. Approximately 10% of the insects with these composite vectors had mosaic eyes when transposase was present. Germline excision rates of approximately 0.04% were observed in these same insects. Again, these values are considerably less than those reported for uninterrupted elements. In addition to the potential importance of subterminal sequence spacing (Lozovsky *et al.*, 2002), Lohe and Hartl (2002) suggested that efficient mobilization of *mariner in vivo* also depends on the presence of critical sequences located quite distant from the ITRs. Based on the mobility characteristics of about 20 *mariner* elements with a wide range of internal deletions, they concluded that there are three regions within the element that play an important role in *cis*. Region I is approximately 350 bp in length and is located 200 bp from the left 5′ ITR. Region II is approximately 50 bp in length and located approximately 500 bp from the right 3′ ITR. Region III is about 125 nucleotides in length and located approximately 200 bp from the right ITR (Lohe and Hartl, 2002). While the presence of subterminal sequences that play a critical role in the movement of many Class II transposable elements is not unusual, what is uncommon in the case of *mariner* is the location of these *cis*-critical sequences. Their dispersed distribution within the element is unique, and consequently, manipulating the element for the purposes of creating gene vectors and associated tools without disrupting these important relationships may be difficult.

The postintegration mobility of *Mos1* can also be regulated by nonstructural aspects of the system including "overproduction inhibition" and "dominant-negative complementation." Increasing the copy number of *Mos1* in the genome resulted in a 25% decrease in the rate of germline excision. Copy number increases in *Mos1* presumably lead to increased transposase levels and, by an unknown mechanism, to the inhibition of excision (Lohe and Hartl, 1996b). High concentrations of transposase may lead to nonspecific associations of the protein resulting in inactive oligomers of transposase. In addition, the presence of mutated forms of *Mos1* transposase can repress the activity of functional transposase. Because the transposases of other transposable elements act as dimers or multimers it is thought that mutated *Mos1* transposases may become incorporated into multimers with functional transposases, thereby inactivating the entire complex (Lohe and Hartl, 1996b).

The possibility that transposase overproduction may negatively affect its own activity is a highly important concept in terms of vector system development. Most systems have the helper transposase under strong promoter regulation to optimize transpositional activity, though this may, indeed, be counterproductive. For *mariner* vectors, and potentially other systems, optimal transformation may require testing various helper promoters and a range of plasmid concentrations.

The postintegration mobility properties of *mariner* were also examined in *A. aegypti* (Wilson *et al.*,

2003). As part of an effort to create an enhancer trapping and gene discovery technology for *A. aegypti*, they created nonautonomous *mariner*-containing lines and lines expressing *Mos1* transposase. By creating heterozygotes between these two lines, they attempted to detect and recover germline transposition events, but only a single germline transposition event was recovered after screening 14 000 progeny. Somatic transpositions were detected, and while precise estimates of rates of somatic transposition were not possible because of the detection method, the authors observed fewer than one event per individual which they estimated to be an indication of a very low rate of movement. The vectors used by Wilson *et al.* (2003) resembled the simple vectors reported by Lozovsky *et al.* (2002) which had apparently disrupted spacing of the ITRs, and partial deletions of *cis*-critical sequences described by Lohe and Hartl (2002).

While the postintegration stability of *mariner* has been described in two species and appears to be a general mobility characteristic of this element, and not a reflection of a species-specific host effect, paradoxical observations remain to be explained. First, the use of *mariner* as a primary germline transformation vector in non-drosophilid insects and in non-insect systems is an effective means for creating transgenic organisms. Indeed, the host range of *mariner* as a gene transformation vector is unrivaled by any of the other gene vectors currently employed for insect transformation. *mariner* has been used as a gene vector in microbes, protozoans, insects, and vertebrates. The rates of germline transformation using *mariner*-based vectors in insects is approximately 10% or less, and is comparable to the efficiency of *Hermes*, *Minos*, and *piggyBac* gene vectors. This raises the question of whether *mariner* vectors present on plasmids behave the same as *mariner* vectors integrated into insect chromosomes. Given the rates of germline integration from plasmids it appears that the *mariner* vectors being used are not suffering from "critical spacing/critical sequence" defects. In addition, the *in vitro* behavior of *mariner* also differs from the behavior of chromosomally integrated elements. Tosi and Beverly (2000) demonstrated that only 64 nucleotides from the left end, and 33 nucleotides from the right end, of *mariner* were essential for transposition of a 1.1 kb vector *in vitro*. The rate of transposition of a minimal *mariner* vector *in vitro* was only two-fold less than that of a vector containing essentially a complete *mariner* element. These results suggest that *mariner* mobility has relatively simple sequence requirements and that the role of subterminal sequences is minimal *in vitro*. These apparently conflicting data suggest that host factors may play an important role in the transposition process *in vivo*, and may influence the relative importance of *cis* sequences in the *mariner* transposition process. The broad distribution of *MLE*s and host range of *mariner/Mos1* suggest, however, that host factors play little role in the movement of these elements.

The postintegration behavior of *mariner/Mos1* seems to indicate that this element will not be a good candidate for developing gene-finding tools such as promoter/enhancer trapping and transposon tagging systems in *A. aegypti* or perhaps other insects. On the other hand, if a high level of post-integration stability is desired, then *mariner* is an appropriate element to consider in insects. The potential of this element to be lost through excision or transposition is low, even in the presence of functional *mariner* transposase. As currently configured and used, *mariner* vectors may be considered as suicide vectors in insects since they essentially become dysfunctional upon integration.

10.5.3.5. *MLE*s have been found in other insects While hundreds of *MLE*s have been described, few have been shown to be functional. The original *mariner* element from the *white*-peach allele was transpositionally competent although it did not produce a functional transposase. *Mos1* is a functional autonomous element and has been the basis for constructing all *mariner* gene vectors that function in insects. *Himar1* is a functional element from the *irritans* subgroup that was reconstructed based on multiple sequence comparisons of elements within this group. It has not been shown to be functional in insects despite significant efforts to do so. Lampe *et al.* (2000) report that at least eight other elements from the other subgroups are likely to be active or made active by minor modifications.

10.5.4. *Minos*

The first germline transformation of a non-drosophilid insect mediated by a transposon-based vector system was achieved with the *Minos* element. *Minos* was originally isolated as a fortuitous discovery in *D. hydei* during the sequencing of the non-coding region of a ribosomal gene (Franz and Savakis, 1991). *Minos* was found to be a 1.4 kb element having, unlike the other Class II transposons used as vectors, relatively long ITRs of 255 bp, with its transcriptional unit consisting of two exons (**Figure 1**). Additional *Minos* elements were isolated from *D. hydei* having small variations of one or two nucleotides, though the new elements had a transition change that restored the normal reading frame allowing translation of a functional transposase.

The sequence homology, general structure, and TA insertion-site specificty placed *Minos* within the *Tc* transposon family (Franz *et al.*, 1994). *Minos* was first used to transform *D. melanogaster* with *Minos*-mediated events demonstrated by sequencing insertion sites and remobilization of integrations (Loukeris *et al.*, 1995a). The first non-drosophilid transformation with *Minos* was achieved in a medfly *white eye* host strain using a cDNA clone for the medfly *white* gene as a marker (Zwiebel *et al.*, 1995), at an approximate frequency of 1–3% per fertile G_0 (Loukeris *et al.*, 1995b). *Minos* transposition was subsequently demonstrated in dipteran and lepidopteran cell lines (Klinakis *et al.*, 2000; Catteruccia *et al.*, 2000a), with germline transformation reported for *Anopheles stephensi* (Catteruccia *et al.*, 2000b) and *D. virilis* (Megna and Cline, personal communication). Recently transformation frequencies have been substantialy increased in *Drosophila* and medfly by the use of *in vitro* synthesized transposase mRNA as helper (Kapetanaki *et al.*, 2002).

Although *Minos* has not been widely used for insect transformation, embryonic and cell line mobility assays in several insect species in the Diptera, Lepidoptera, and Orthoptera have indicated a broad range of function. Notably, *Minos* transposition in the cricket *Gryllus bimaculatus* was driven by transposase regulated by a *Gryllus* actin gene promoter, and not by the *Drosophila hsp70* promoter that has been widely used in dipterans (Zhang *et al.*, 2002). The broad function of the *Minos* vector is further supported by its ability to transpose in a mouse germline (Drabek *et al.*, 2003).

Minos structure places it within the *mariner/Tc* transposon superfamily, though knowledge of the distribution of *Minos* is thus far limited to the genus *Drosophila* (Arca and Savakis, 2000). In *Drosophila*, *Minos* is clearly widely distributed in the *Drosophila* and *Sophophora* subgenera, though discontinuously in the *Sophophora*. As noted for the *hAT*, *mariner*, and *piggyBac* elements, *Minos* may have also undergone horizontal transfer between *Drosophila* species.

10.5.5. *Tn5*

Tn5 is one of a number of very well-characterized transposable elements from prokaryotes. Recently, hyperactive forms of this element have been created in the laboratory that have proven to be the basis for the development of a number of commercially useful genomics tools (Goryshin and Reznikoff, 1998; Epicentre, 2004). *Tn5*-based genomics tools can be used in a wide variety of bacterial species and given the system's independence from host-encoded factors, might be applicable to eukaryotic systems as well (Goryshin *et al.*, 2000). Efforts to use *Tn5* as an insect gene vector have been successful.

Tn5 is a prokaryotic transposon 5.8 kb in length, and it is often referred to as a composite transposon because it consists of five independently functional units (review: Reznikoff, 2000) (**Figure 1**). It contains three antibiotic resistance genes that are flanked by 1.5 kb inverted repeat sequences. Each inverted repeat is actually a copy of an *IS50* insertion sequence that are themselves functional transposons. Each *IS50* element contains 19 bp terminal sequences known as outside end (OE) and inside end (IE), and while OE and IE are very similar, they are not identical. *IS50* also encodes for two proteins: transposase (Tnp) is 476 amino acids long and catalyzes transposition while the second protein is an inhibitor of transposition (Inh). The *IS50* elements present at each end of *Tn5* are not identical and only *IS50R* is fully functional. *IS50L* contains an ochre codon that prematurely terminates the Tnp and Inh proteins resulting in a loss of function of both proteins.

The transposition reaction and all of the components involved in the reaction have been studied in great detail (Reznikoff *et al.*, 1999). Transposition proceeds by a cut-and-paste process involving binding of Tnp to the end sequences followed by dimerization of the bound Tnp to form a synaptic complex. Cleavage at the ends of the element results in an excised transposon with bound transposase that interacts with a target DNA molecule. Strand transfer results in the integration of the element into the target, and *in vitro*, this reaction requires only a donor element, a target DNA molecule, transposase, and Mg^{2+} (Goryshin and Reznikoff, 1998). Modifications of both the transposase and the terminal 19 bp sequences have lead to the creation of *Tn5* elements consisting of little more than two copies of end sequences that can be mobilized a 1000-fold more efficiently than an unmodified *Tn5* element. This hyperactive *Tn5* system has been developed into a powerful tool for genetic analysis of a variety of organisms. *Tn5* has been attractive as a broad host range genomics tool because its pattern of integration is random and its biochemical requirements very simple. *Tn5* has been shown to function in a variety of bacterial and nonbacterial systems.

Current insect transformation protocols consist of microinjecting a mixture of two plasmids into preblastoderm embryos (see Section 10.6.3). One plasmid contains a nonautonomous transposable element with the transgenes and genetic markers of interest while the second plasmid contains a copy of the transposase gene. Transient expression of the

transposase gene is required postinjection and is followed by element excision and integration. Previous experiments examining the frequency of element excision of elements, such as *Hermes*, *mariner*, *Minos*, and *piggyBac*, from plasmids injected into insect embryos along with helper plasmids indicated that only one plasmid per thousand injected underwent an excision event. Therefore, 99.9% of the donor plasmids introduced into insect embryos will contribute nothing to the transformation efforts. The introduction of preexcised elements configured as active intermediates, such as synaptic complexes, was considered a means to permit higher integration rates and overall efficiency of transformation.

Transgenic *Aedes aegypti* were created using a *Tn5* vector containing DsRed under the regulatory control of the 3xP3 promoter (Rowan *et al.*, 2004). Preexcised vectors in the form of synaptic complexes were injected into preblastoderm embryos. Nine hundred adults were obtained from the injected embryos and families consisting of approximately 10 G_0 individuals were established. Two families of G_0 individuals produced transgenic progeny for an estimated transformation frequency of 0.22% (2/900). Analysis of the transgenic progeny showed that multiple integrations of *Tn5* occurred in each line. The patterns of integrations were complex with evidence of the *Tn5* vector integrating into *Tn5* vector sequences. The integration of the vector into copies of itself followed by the integration of the resulting concatamers was very unusual, and in no case was a simple cut-and-paste integration of the *Tn5* vector found with characteristic 9 bp direct duplications flanking the element. The complex pattern of *Tn5* integration was thought to be a direct consequence of injecting preassembled intermediates, that were inactive in the absence of Mg^{2+}. Therefore, as soon as the synaptic complexes were injected they became activated and the first target sequences the elements were likely to encounter were other *Tn5* synaptic complexes. At the time of injection, *A. aegypti* embryos only contain approximately four to eight nuclei making genomic target DNA relatively rare. Furthermore, the synaptic complexes injected were expected to have a very short half-life. Therefore, although active intermediates were being introduced, a number of factors contributed to the inefficiency observed with this system including a short half-life of the active intermediate and low numbers of genomic target sequences. Injecting binary plasmid systems (as is done with *Hermes*, *mariner*, *Minos*, and *piggyBac*), while relatively inefficient in producing active transposition intermediates, achieves persistence over an extended period of time. Consequently, more target genomes are exposed to active vectors over a longer period of time, resulting in higher transformation rates. The limitations of injecting synaptic complexes is unlikely to be specific to the *Tn5* system and similar approaches with other insect gene vectors are likely to encounter similar problems. It should be noted, however, that the results of Rowan *et al.* (2004) demonstrate that *Tn5* is functional in insects and, while injecting active intermediates is not recommended, using *Tn5* in a more conventional binary plasmid system consisting of a donor and helper plasmids is likely to be a viable option for creating transgenic insects.

10.6. Transformation Methodology

The technical methodology for insect transformation has largely remained the same or only slightly modified from the techniques originally used to transform *Drosophila*. The references cited for *P* transformation are relevant to this, as well as several recent articles that focus on methods for non-drosophilid transformation (Handler and O'Brochta, 1991; Morris, 1997; Ashburner *et al.*, 1998; Handler, 2000; Handler and James, 2000). The most variable aspect of this method is the preparation of embryos for DNA microinjection, though arguably, the lack of new techniques for DNA introduction has been the primary limitation in the more widespread use of the technology. While all successful insect transformations have utilized microinjection, variations on this method have been necessary for different types of embryos, and most of the procedures must be tested empirically and modified for particular insect species. This may be extended to different strains and for a variety of local ambient conditions including temperature and humidity. The apparatus for microinjection is usually the same for all species, though a wide variety of variations and modifications are possible and sometimes required. The basic equipment includes an inverted microscope or a stereozoom microscope with a mechanical stage having a magnification up to 60 to 80×; a micromanipulator that is adjustable in three axes with an appropriate needle holder; and a means to transmit the DNA into the egg. For dechorionated eggs, transmitted light allows precise positioning of the needle within the egg posterior, while direct illumination is needed for nondechorionated eggs that typically include mosquitoes and moths.

The standard for gene transfer methodology in general, and embryo microinjection in particular, was originally developed for *Drosophila*. The standard method involves collecting preblastoderm embryos

within 30 min of oviposition, and dechorionating them either manually or chemically. The timing of egg collection and DNA injection is related to the need to inject into preblastoderm embryos during a phase of nuclear divisions previous to cellularization. This allows the injected DNA to be taken up into the nuclei, and specifically into the primordial germ cell nuclei that are the gamete progenitors. For *Drosophila*, cellularization of the pole cells begins at approximately 90 min after fertilization at 25 °C, with blastoderm formation occurring about 30 min afterwards. The timing of these events and location of the pole cells varies among insects, and thus some knowledge of early embryogenesis in the desired host insect is highly advantageous. In the absence of this information for a particular species, the most prudent time of injection would be the earliest time after oviposition that does not compromise viability.

10.6.1. Embryo Preparation

Manual dechorionation of *Drosophila* eggs is achieved by gently rolling the eggs on double-stick tape with a forcep until the chorions peel off. While gentle on the eggs and requiring little desiccation time, manual dechorionation is tedious and has not been applicable to any other insect. Chemical dechorionation is typically achieved by soaking eggs in a 50% bleach solution (2.5% hypochlorite) for 2–4 min and washing at least three times in 0.02% Triton X-100. Tephritid fruit fly eggs usually have thinner chorions that can be dechorionated in 30% bleach (1.25% hypochlorite) in 2–3 min, but this must be determined empirically since they are easily overbleached resulting in death, either directly or after injection. Some species, such as *M. domestica*, can be only partially dechorionated, but bleached eggs can be released from the chorion by agitation. We have found the simplest and most precise method for bleach dechorionation with rapid washes is by using a 42 mm Buchner funnel with a filter flask attached to a water vacuum. Eggs can be washed into the funnel on filter paper and swirled within the funnel with the solution gently sucked out by regulating the water flow or the seal between the funnel and flask. The last wash is done on black filter paper that allows the eggs to be easily detected, which facilitates their mounting for injection (see below).

Many insects eggs cannot be dechorionated without a high level of lethality, and must be injected without dechorionation. These include most moth and mosquito species. *Drosophila* and tephritid flies can, similarly, be injected without dechorionation, and while embryo viability after injection is often lower than for dechorionated eggs, the frequency of transformation in surviving embryos is often higher. It is more difficult to determine a precise site for injection in nondechorionated eggs, though this can be aided by adding food coloring to the DNA injection mix.

After dechorionation, fruit fly embryos are typically placed on a thin strip (~1 mm) of double stick tape placed on a microscope slide or 22 × 30 mm cover slip, though use of a cover slip is more adaptable for subsequent operations. A thin strip of tape is suggested due to anecdotal reports of toxic solvents from the tape affecting survival, though some particular tapes are considered to be nontoxic (3M Double Coated Tape 415; 3M, St. Paul, MN, USA) and some are useful for particular applications such as aqueous conditions needed for mosquito eggs. Adhesives resistant to moisture include Toupee tape (TopStick^{TM}, Vapon Inc.) and Tegaderm (3M). When eggs are injected under oil, the tape strip is placed within a thick rectangle created with a wax pencil that can retain the oil. It is important that the wax fence not be breached by oil when overlaying the eggs, since the loss of oil will result in embryo death.

Where possible, eggs are placed on the tape in an orientation having their posterior ends facing outwards towards the needle, but at a slight angle. All fruit fly eggs must be desiccated to some extent before injection. The interior of the egg is normally under positive pressure, and yolk and injected DNA will invariably flow out after injection without desiccation. This will result in lethality, sterility (from loss of pole plasm), or the lack of transformation if the plasmid DNA is lost. The time and type of desiccation, however, must be evaluated empirically, and sometimes varied during the course of an injection period. A major factor for dechorionated eggs is the length of time they are kept on moist filter paper before being placed on the tape. Typically we desiccate embryos on one strip of tape (15–20 embryos) for 8 to 10 min. Depending on the ease of injection the time can be varied by 1–2 min. In ambient conditions that are humid, it may be necessary to desiccate in a closed chamber with a drying agent (e.g., drierite), with or without a gentle vacuum. An important consideration is that a very short variation in the time for desiccation can be the difference between perfect desiccation and overdesiccation resulting in death, and that the optimal desiccation time will vary for different eggs on the tape. Thus, it is unlikely that all the eggs will respond well to the set conditions, which must be modified so that the majority of eggs can be injected with DNA at a high level of survival and fertility. After the determined time for desiccation, the eggs

must be placed immediately under Halocarbon 700 oil, or oil of similar density, to stop the desiccation process. Desiccation of most nondechorionated eggs is more challenging and one approach is to soak eggs in 1 M NaCl for several minutes. In contrast, for nondechorionated mosquito eggs, desiccation can occur within 1–2 min after removal from water, which is evidenced by slight dimpling of the egg surface, and this must be observed to avoid overdesiccation. Due to the rapidity of desiccation, mosquito eggs are typically arranged on moist filter paper and blotted together onto a taped cover slip from above, and after desiccation, the eggs are submerged in Halocarbon oil. Nondechorionated eggs from many species do not require oil, and it may be lethal for some insects such as moths, yet oil submersion was helpful for the survival of *Drosophila* and tephritid flies.

10.6.2. Needles

The type of needle and its preparation is possibly the most important component of successful embryo injections. Most dechorionated fruit fly eggs can be injected easily with borosilicate needles, which are drawn out to a fine tip and broken off to a 1 to 2 μm opening. Opening the tip is typically achieved by scraping the needle against the edge of the slide carrying the eggs to be injected. Opening the needle by beveling, however, creates consistently sharp tips that are much more important for nondechorionated eggs, and stronger alumina-silicate and quartz needles also provide an improvement to easily pierce chorions or tough vitelline membranes. Beveled needles are also critical when a large tip opening is required for large plasmids that are susceptible to shearing. Preparation of borosilicate needles, pulled from 25 μl capillary stock that has been silanized, can be achieved with several types of vertical or horizontal needle pullers, and we find the Sutter Model P-30 (Sutter Instruments, Novato, CA, USA) vertical micropipette puller to be highly effective. Alumina-silicate needles, and certainly quartz needles, require more sophisticated pullers that allow for fine programmable adjustment of high filament temperatures and pulling force, and the Sutter Models P-97 and P-2000 fulfill this need. Several needle bevelers are available, with the Sutter BV-10 used by many laboratories.

10.6.3. DNA Preparation and Injection

A mixture of highly purified vector and helper plasmid DNA is essential to embryo survival. This is achieved most optimally by purifying plasmid twice through cesium chloride gradients or a solid-phase anion exchange chromatography column. These have the advantage of high yields of DNA, but the disadvantage of specialized equipment and long preparation times. Successful transformation has been achieved with plasmids prepared with silica-gel membrane kits from Qiagen Corp. (Valencia, CA, USA), but their successful use has been inconsistent, with failures possibly related to the type of host bacteria and its growth conditions. The Qiagen Endotoxin-free plasmid preparation systems allow additional purity, and this system is routinely used for successful plasmid injection.

Purified plasmid concentration must be titered accurately and verified by gel electrophoresis previous to injection mix preparation. Appropriate amounts of vector and helper plasmid are ethanol precipitated, washed several times in 70% ethanol, and resuspended in injection buffer. Injection buffer has typically been the same as that originally used for *Drosophila* (5 mM KCl, 0.1 mM sodium phosphate, pH 6.8), though this may not be optimal for other insects and embryo survival should be assessed by control injections. Total DNA concentration for injection should not exceed 1 mg ml^{-1}, using two- to fourfold higher concentration of vector to helper (e.g., 600 ng μl^{-1} vector to 200 ng μl^{-1} helper). Higher DNA concentrations are inadvisable since they are subject to shearing during injection and may clog the needle, and the nucleic acids and/or contaminants can be toxic to the embryo. High transposase levels may also have a negative effect on transposition, as with the overproduction–inhibition phenomenon observed with *mariner* (Lohe and Hartl, 1996b).

Previous to injection the DNA mixture should be filtered through a 0.45 μm membrane, or centrifuged before loading into the injection needle. Typically, DNA is back-filled into the injection needle using a drawn-out silanized 100 μl microcapillary, and a microliter of DNA should be sufficient for injecting hundreds of eggs.

10.6.3.1. DNA injection The microinjection of DNA into embryos requires a system that forces a minute amount of DNA through the needle in a highly controllable fashion. Remarkably, many *Drosophila* laboratories simply use a mounted syringe and tubing filled with oil connected to a needle holder, with manual pressure applied. This system is successful due to accumulated expertise and the efficiency of transformation in the species, but would probably be less useful for injecting more sensitive embryos that transform less easily. Regulated air-pressure systems are available that are economical and allow highly controlled and rapid DNA injection. We use the PicoPump from WPI that

is most versatile in allowing positive and negative (with vacuum) pressure, and a hold capability that prevents back flow into the needle resulting in clogging (especially by yolk). A less expensive system can be constructed from Clippard components (Clippard Instrument Laboratory, Inc., Cincinnati, OH, USA) that uses a simple air-pressure regulator and electronic valve and switch (see Handler, 2000). Needle holders from WPI can be used with both systems (MPH-3 and MPH-1, respectively).

All embryo injections are performed on a microscope with a mechanical stage, with the injection needle mounted on a micromanipulator. Microscopes first used for *Drosophila* transformation were inverted or compound microscopes, but the availability of a useful mechanical stage and stage adaptor for the Olympus SZ stereozoom microscopes makes this the most versatile choice (the Olympus stage can be mounted on most stereomicroscopes). The micromanipulator can be free-standing next to the stage or mounted on the microscope base. It allows the precise positioning of the needle at the desired point of entry into the egg, while the actual injection occurs by using the mechanical stage to push the egg onto the needle. Piezo Translators that were developed for rapid and automatic intracellular injection may be more efficient for some embryos, and will obviate the need for a mechanical stage (Peloquin *et al.*, 1997). The WPI MPM20 translator used with the PV820 PicoPump allows a fully automated system for egg penetration, DNA injection, and needle withdrawal.

10.6.4. Postinjection Treatment

After injection the cover slip can be placed in a covered petri dish (but not sealed) with moist filter paper. The use of square dishes with black filter paper seems to be most suitable for up to six cover slips and simple observation of the embryos and hatched larvae. For injected embryos submerged in oil, oxygen concentration may be a limiting factor for development, if not viability. This can be ameliorated by reducing the crowding of eggs on the cover slip, or by incubation in a portable hatbox tissue culture chamber that is humidified and under slight positive pressure with oxygen. For eggs without oil, oxygen saturation without pressure is advisable.

Most helper constructs have the transposase gene under heat shock regulation. The *Drosophila hsp70* promoter is a constitutive promoter that is active in the absence of heat shock (but also responds to anoxia which may occur in embryos under oil), and transformation is possible with most vectors with or without heat shock treatment. If heat shock is desirable, it should be noted that the optimal temperature varies for different species. For example, *hsp70* responds optimally at 37 °C in *Drosophila*, but at 39 °C in medfly (Papadimitriou *et al.*, 1998). Injected embryos should be incubated for at least 4–6 h after injection before heat shock, or after overnight incubation. Optimal temperatures for insect development vary, but the lowest temperatures possible can be beneficial to survival, and the injection process can slow development by 50% or more. Thus, larval hatching may be delayed considerably and hatching should be monitored for several days after the expected time before discarding embryos.

Hatched larvae can be placed on normal culture media, though they may be weak and require careful handling and soft diet. Rearing of putative transgenic lines is typically achieved by backcrossing to the parental line in small group matings, or individual mating if a determination of transformation frequency is required. Inbreeding of G_0s can minimize rearing efforts, but this may be complicated by high rates of infertility which is typically close to 50% after fruit fly injections.

10.7. Research Needs for Improved Transgenesis

10.7.1. DNA Delivery

Dramatic progress has been made in transformation technology for non-drosophilid insects, and it appears that the vectors and markers in use should be widely applicable. Nevertheless, transformation of many other insect species will be highly challenging, primarily due to limitations in the delivery of DNA into preblastoderm embryos. As noted, to date all successful non-drosophilid transformations have resulted from embryonic microinjection of DNA, but for many species current injection techniques are likely to result in high levels of lethality or sterility. Experimentation with alternative methods has been reported, though arguably, none has been tested exhaustively for germline transformation, or vector systems were used that are now known to be ineffective. The most promising method is biolistics where eggs are bombarded with micropellets encapsulated by DNA, which was first developed as a ballistics method to transform plant cells (Klein *et al.*, 1987). Ballistics is based upon a "shotgun" technique for bombardment, and it is the only non-injection method successfully used to transform an insect. This was a *P* transformation of *Drosophila*, though only a single transformant line was created

and the technique never gained wide applicability (Baldarelli and Lengyel, 1990). This was most likely due to the high efficiency of *P* transformation of *Drosophila* by microinjection, eliminating the need for an alternative technique. Mosquito eggs are considerably more difficult to inject, and a significant effort was made to modify a biolistics approach to DNA delivery in *A. gambiae*, using a burst of pressurized helium for bombardments (Miahle and Miller, 1994). This technique was effective in introducing plasmid DNA into mosquito eggs, yielding *high* levels of transient expression of a reporter gene. Biolistics was subsequently used for transient expression in specific tissues, allowing the testing of fibroin gene promoters in the *B. mori* silk gland (Horard *et al.*, 1994; Kravariti *et al.*, 2001). Recent advances have included the use of a rigid macrocarrier in the Bio-Rad PDS/1000-Helium biolistics apparatus, which minimizes the blast effect in soft tissue (Thomas *et al.*, 2001). This allows greater micropellet penetration into insect tissues with improved survival. Despite these advances in delivering DNA into eggs and tissue, biolistics has yet to yield a germline transformant.

The only other method reported for DNA delivery is electroporation, which, like biolistics, has resulted in high levels of transient expression of plasmid-encoded genes in *Drosophila* (Kamdar *et al.*, 1992), as well as in *Helicoverpa zea* and *M. domestica* (Leopold *et al.*, 1996). Though transformation has not been reported, as with biolistics, it is not apparent that this was seriously tested or if functional vectors systems were used (certainly for non-drosophilids). Electroporation techniques have also advanced in recent years, with DNA transferred into many different tissue types from a variety of organisms using new electroporation chamber designs and electric field paramenters.

These recent advances with both biolistics and electroporation are highly encouraging that new efforts will have greater chances for success, and they deserve a high priority for testing. Both methods also have the advantage, if successful, of delivering DNA simultaneously to multiple embryos, ranging from hundreds to thousands depending on the species. This would be highly beneficial to all transformation experiments, but especially so for species that transform at low frequencies. These methods could also be used in cellularized embryos after blastoderm formation in insects having embryos that cannot be handled easily or collected in the preblastoderm stage.

Other approaches to DNA delivery can include the incorporation of vector/helper DNA into bacterial or viral carriers, that may be delivered by maternal injection or feeding. Variations on microinjection that might be required for ovoviviparous insects include maternal injection into ovaries or abdominal hemocoel (Presnail and Hoy, 1994), and the use of liposomes might allow injection into cellularized embryos (Felgner *et al.*, 1987). All of these techniques should be reevaluated with the use of vectors and markers now known to be highly efficient in non-drosophilid systems.

10.7.2. Gene Targeting

The ability to target genes to specific or desired integration sites in the genome would be highly advantageous to the basic and applied uses of transgenic strains. The expression of transgenes in most vector integrations is affected negatively by chromosomal position effects, so target sites known to be devoid of, or insulated from, suppression elements could be utilized for optimal or consistent transgene expression. Target sites positioned in innocuous genomic regions could also eliminate random integrations into genes necessary for viability and fertility, eliminating costs to fitness in host strains. It will also be highly important to gene expression studies to achieve reliable methods for gene replacement or targeted transposition, which is especially important for systems where preexisting null mutations, or gene "knockouts," do not exist.

Gene targeting can be achieved, generally, in two ways. First is homologous recombination where an endogenous genomic sequence is replaced by recombination with homologous sequences within or surrounding the transgene. This results in targeted transposition which can be used for gene replacement, or for targeting to an innocuous genomic region. This approach has been effective in transforming lower eukaryotes, and plant and vertebrate systems (see Bollag *et al.*, 1989) and has been reported to occur in *Drosophila* (Cherbas and Cherbas, 1997) and mosquito (Eggleston and Zhao, 2000) cell line studies. Homologous recombination can also occur in insects *in vivo*, but this is not routine and thus far, must be facilitated. In *B. mori*, female moths were infected with a modified AcNPV baculovirus that had its polyhedrin gene replaced with fibroin light chain-GFP gene fusion (Yamao *et al.*, 1999). Progeny of the infected moths exhibited stable integration of the gene fusion into the genomic fibroin gene, with resulting GFP expression. This particular method relies on host susceptibility to baculovirus infection, though conceivably other pantropic or species-specific viruses could be used for a wider range of insects.

Homologous recombination was also achieved in *Drosophila* where linearized extrachromosomal

DNA was found to be recombinogenic with homologous sequences in the genome (Rong and Golic, 2000). This was achieved by using the *FRT*–FLP recombination system to create DNA circles, which were linearized at a rare endonuclease recognition site within the *FRT* sequences, resulting in a *yellow* marker gene integrating into its homologous chromosomal site. This system has potential application in any insect species that can be stably transformed, but it requires the integration of three components which can be tedious to achieve and may present fitness costs to the host. Though its use could have major importance to genetic studies in non-drosophilid insects, thus far it is not routinely used in *Drosophila*.

10.7.2.1. Site-specific recombination Site-specific recombination systems such as the *FRT*–FLP system from the 2 μm circle of yeast (Senecoff *et al.*, 1985) mentioned above, and the bacteriophage Cre/*lox* system (Hoess *et al.*, 1985) can be used for various types of gene targeting and chromosomal manipulation. Both systems function in *Drosophila* in which recombination occurs between specific sequences in the presence of a recombinase enzyme (Golic and Lindquist, 1989; Siegal and Hartl, 1996). For *FRT*, the recombination site consists of two 13 bp inverted repeats separated by an 8 bp spacer that specifically recombines with identical *FRT* sites in the presence of FLP recombinase. Depending upon the orientation of the *FRT* sites, the intervening sequence between them can be inverted or deleted by recombination (Golic and Golic, 1996; Golic *et al.*, 1997). When placed within a vector, such *FRT* rearrangements can allow several types of vector manipulation after genomic integration. Genes or sequences within the vector necessary for the initial transformation or selection, but deleterious to use of the transformed strain, can be deleted or inactivated (Dale and Ow, 1991; see Handler, 2002b). This may include the marker system used for selection (e.g., chemical resistance system) or even a transposase gene used in a single plasmid autonomous vector system. Expression of genes of interest can be similarly manipulated by placing *FRT* sites outside the gene and within an internal noncoding region. Of particular importance to transgene stability would be the rearrangement of vector sequences required for mobility, that typically would include the terminal and subterminal ITRs. If *FRT* site placement between the terminal sequences does not hinder the primary transposon vector integration, then subsequent subterminal ITR deletion could eliminate any secondary vector mobilization. Such mobilization or cross-mobilziation would have serious consequences for strain stability and function, as well as ecological risks due to unintended transmission of the vector into other organisms. Eliminating this possibility would provide a major advancement to the applied use of transgenic insects.

The ultimate use of recombination systems for improved transformant stability and transgene expression would be their development into a second generation of vectors that use an integrated recombination site as a stable chromosomal target. Plasmids having the same recombination site and a marker gene would be used as vectors that integrate by recombination in the presence of recombinase. The expectation is that these systems would be highly stable in eukaryotes, and specific target site loci could be selected that are minimally affected by position effect variegation/suppression. The internal 8 bp spacer sequence within the *FRT* can be varied, but only identical *FRT* sequences will recombine with one another. Thus, multiple independent *FRT* target sites can be incorporated into the same genome. Importantly, manipulations by *FRT* recombination will depend upon a controllable source of FLP recombinase, which can be provided as a separate transgene integration, or exogenously by DNA, RNA, or protein injection.

10.8. Summary

After concerted efforts for more than 30 years to achieve gene transfer in non-drosophilid insects, only in the last decade have these efforts been fruitful. Since 1995 the germline of nearly 20 species in four orders of insects have been transformed, and this number may be only limited by the insects of current experimental and applied interest. Unlike plant and vertebrate animal systems that allow relatively efficient genomic integration of introduced DNA, insect systems have generally relied on vector-mediated integrations, and the only vectors found reliable for germline transformation are those based on transposable elements. Curiously, the two main vector systems developed for routine use in *D. melanogaster*, and originally discovered in that species, *P* and *hobo*, have not been applicable as vectors to any other species. Yet, four other transposons found in non-*melanogaster* or non-drosophilid species are widely functional in insects, and for some, other organisms. Their discovery has been of enormous importance to the wider use of transformation technology, since little progress would have been made if most vector systems were specific to a particular host. Equal in importance to the advancements in vector development, have been concurrent progress in genetic marker discovery and development. This began with the finding

that cloned eye color genes from *Drosophila* could complement existing mutations in other insects, and has continued with the more recent use of several fluorescent protein genes that are widely applicable as markers for transformation and reporters for gene expression.

The advancement of these techniques comes at a fortuitous time when genomics is providing a wealth of genetic infomation and resources that might be used to create transgenic strains of pest and beneficial insects to control their population size and behavior. As part of these efforts, genetic transformation is also critical to functional genomics studies that will provide information essential to understanding the biological function of genetic material, and relating specific genomic elements to those functions. Techniques such as enhancer traps and transposon tagging, which rely on remobilizable insertional mutagenesis, are only possible with *trans*poson-based vector systems, and other techniques such as RNA interference (RNAi) are greatly facilitated by these systems. Together, routine methods for transposon-mediated germline transformation and genomics analysis should provide the tools for dramatic progress in our understanding and control of insect species.

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